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-PhD Thesis-

The Open University

TIGEM

**Telethon Institute of Genetics and Medicine
(Naples, Italy)**

Life and Biomolecular Sciences

GENE THERAPY

FOR INBORN ERRORS OF METABOLISM

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TABLE OF CONTENT

ABSTRACT	4
 <i>1.BACKGROUND</i>	
1.1 Inborn errors of metabolism	10
1.2 Disease candidates for liver-directed gene therapy	11
 <i>2. INTRODUCTION</i>	
2.1 Crigler-Najjar Syndrome	17
2.2 Bilirubin	19
2.3 Bilirubin toxicity	21
2.4 UCB Glucuronidation	22
2.5 The UGT family	26
2.6 Molecular defect in CN type I syndrome	29
2.7 Current Therapies for Crigler-Najjar Syndrome	30
2.8 Animal models of Crigler-Najjar syndrome type I	31
2.9 Gene therapy for Crigler-Najjar syndrome type I	34
 <i>3. SPECIFIC AIMS</i>	 37
 <i>4. MATERIALS AND METHODS</i>	
4.1 Construction and production of AAV vectors	40
4.2 Animal experiments	42
4.3 Bilirubin determinations	44

4.4 Western blot analysis, enzyme assay, and qPCR on tissues	44
4.5 GFP expression in muscle	46
4.6 Statistical analysis	47

5. RESULTS

5.1 Sustained reduction of hyperbilirubinemia and excretion of conjugated bilirubin in bile and urine following muscle-directed AAV mediated gene transfer in the Gunn rats	48
5.2 Expression of functionally active UGT1A1 protein in Gunn rat muscles	51
5.3 Vector biodistribution in intramuscularly injected Gunn rats	55
5.4 Evaluation of potency of liver specific expression cassettes <i>in vivo</i>	58

6. DISCUSSION AND CONCLUSIONS

6.1 Muscle-directed gene therapy for Crigler-Najjar syndrome type I	65
6.2 Liver-directed gene therapy for CN type I with AAV2/8	69

7. LITERATURE CITED	74
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ABSTRACT

Inborn errors of liver metabolism are frequent causes of morbidity and mortality especially in children. For several of these diseases, treatment approaches depend on manipulation of the affected metabolic pathway by diet, drugs, vitamin cofactors, enzyme induction, end-product replacement, and alternative pathway activation. Unfortunately, these approaches often remain unsatisfactory especially in the face of illness or catabolism. Ideally, transfer of the normal genes in the liver cells that are defective might restore the metabolic function. The goal of my PhD thesis was to develop gene-based therapeutic strategies to correct a life-threatening inborn error of liver metabolism, Crigler-Najjar syndrome type I (CNI). CNI is a severe inborn error of bilirubin metabolism due to mutations of the uridine diphospho-glucuronosyl transferase 1A1 (*UGT1A1*) gene. Affected patients have elevations of serum bilirubin, and they have to spend extended hours under bilirubin lights throughout childhood and adolescence. Despite this therapy, they remain at risk of brain damage when intercurrent infections may increase production of bilirubin above that which can be controlled by the bilirubin light therapy. Thus, patients with CNI often are advised to consider liver transplantation. Therefore, alternative therapies are highly needed to overcome the mortality and morbidity associated with transplantation procedure, and risks of life-long immunosuppression. Gene therapy has the potential to provide a definitive cure for patient with CNI. My studies have focused on the development of gene therapy strategies for this disease. First, I investigated in Gunn rats, the animal model for CNI, the efficacy of adeno-associated viral (AAV) vector-mediated muscle-directed gene therapy and I found that serotype 1 AAV vector expressing UGT1A1 resulted in expression of UGT1A1 protein and functionally active enzyme in injected muscles, and a

50% reduction in serum bilirubin levels for at least 1 year post-injection. Taken together, these data show that clinically relevant and sustained reduction of serum bilirubin levels can be achieved by simple and safe intramuscular injections.

Following initial problems with intravenous injections of AAV2 vector, a major success has been achieved with AAV2/8 vectors for liver-directed gene therapy of hemophilia. Encouraged by these results and by the possibility of achieving full correction of the hyperbilirubinemia with systemic delivery, next I focused on the design and optimization of an AAV2/8 vector for liver-directed gene therapy of CNL. I generated multiple expression cassettes expressing the UGT1A1 gene inserted into the AAV2/8 vectors for *in vivo* testing. The results of these studies showed that AAV2/8 vector with codon optimized UGT1A1 gene under the control of the hepatocyte-specific LP1 promoter resulted in improved and sustained correction of hyperbilirubinemia in Gunn rats. Taken together, these data demonstrate the development of an optimal expression cassette for liver-directed gene therapy of CNL and form the preclinical basis for the development of a gene therapy trial for this severe disorder.

LIST OF ABBREVIATIONS

AAT Alpha-1-Antitrypsin

AAV Adeno-Associated Virus

Ad Adenovirus

ATP Adenosine Triphosphate

BGH Bovine Growth Hormone

BIND Bilirubin Induced Neuronal Dysfunction

CMV Cytomegalovirus

CN Crigler-Najjar syndrome

CPK Creatine Phosphokinase

ER Endoplasmic Reticulum

ERT Enzyme Replacement Therapy

FBS Fetal Bovine Serum

FH Familial Hypercholesterolemia

FIX Factor IX

GAPDH Glicer aldeide-3-fosfato Deidrogenasi

GC Genome Copies

GFP Green Fluorescent Protein

GSD Glycogen Storage Disease

GSTs Glutathione-*S*-Transferases

HCR Hepatic Control Region

HDAd Helper-Dependent Adenoviral vector

HO Heme oxygenase

HPLC High-Performance Liquid Chromatography

IEM Inborn Errors of Metabolism

IM Intramuscular injections

ITR Inverted Terminal Repeat

IV Intravenous injections

Jj Heterozygous normo-bilirubinemic Gunn rats

jj Homozygous hyperbilirubinemic Gunn rats

KDa Kilo Daltons

LDL low-Density Lipoprotein

LV Lentivirus

MCK Muscle Creatine Kinase

MLV Murine Leukemia Virus

MOI Multiplicity of Infection

MPS Mucopolysaccharidoses

NADH Nicotinammide Adenin-Dinucleotide

NADPH Nicotinamide Adenin-Dinucleotide Phosphate

OLT Orthotopic Liver Transplantation

OTC Ornithine Transcarbamylase

PCR Polymerase Chain Reaction

PFA Paraformaldehyde

PVDF Polyvinylidene Difluoride

RT-PCR Reverse Transcriptase PCR

RV Retrovirus

SDS-PAGE Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis

SV40 Simian Virus 40

TBG Thyroxine Binding Globulin

TSB Total Serum Bilirubin

UCB UnConjugated Bilirubin

UDPGA UDP-glucuronic acid

UDPGA Uridine Diphosphoglucuronate

UDPGT Uridine Diphosphate Glucuronyltransferase

UGT1A1 Uridine Diphospho-Glucuronosyl transferase 1A1

UGTs Uridine Diphosphoglucuronate Glucuronosyl Transferase proteins

WPRE Woodchuck Hepatitis Post-Transcriptional Regulatory Element

1. BACKGROUND

1.1 Inborn errors of metabolism

The term “inborn errors of metabolism (IEM)” was coined by Archibald Garrod in 1908 and is used to describe disorders caused by a deficiency of enzyme catalysis or an enzyme that facilitates the transport of biological substances across membranes. IEM comprise a group of disorders in which a single gene defect causes a clinically significant block in a metabolic pathway that results either in accumulation of substrate upstream enzymatic block or deficiency of the downstream product. Over 200 of these disorders have been identified and a significant proportion of them present clinically in the neonatal period or in early infancy.

The liver is a key organ for most metabolic pathways, and therefore, numerous metabolic inherited diseases originate from defects in this organ (NGUYEN and FERRY 2004). For most conditions, treatment approaches depend on manipulation of the metabolic pathway by diet, drugs, vitamin cofactors, enzyme induction, end-product replacement, and alternative pathway activation. Unfortunately, these approaches are often unsatisfactory particularly when the flux through the affected pathway increases in the face of illness or catabolism (BRUNETTI-PIERRI and LEE 2005). When such relatively simple measures are insufficient to maintain health, exogenous supply of the deficient enzyme, known as enzyme replacement therapy (ERT) can provide quite effective treatment for the enzyme deficiencies (ELLOR *et al.* 2008). For example, patients with lysosomal storage disorders are able to benefit from this type of supplementation. Nevertheless, even when ERT is effective, it does not provide a cure from the disorder, and the patient is dependent on

lifelong intravenous infusion of the drug. For most metabolic disorders, the only permanent treatment available is orthotopic liver transplantation (OLT) (STARZL 1978). A healthy donor liver will restore endogenous enzyme production, effectively repairing the metabolic disorder. However, the transplant procedure is invasive and is associated with high mortality and morbidity. Moreover, OLT requires lifelong immunosuppression that has its own risks. In addition, limited donor organ availability, risks of surgical complications and rejection are major limitations of this treatment. Therefore, it is important to develop alternative treatments to OLT.

Hepatocyte transplantation is an attractive alternative because of the limited invasiveness of the procedure. However, this transplant procedure has several limitations, including poor engraftment and short term efficacy. Moreover, this approach will still require lifelong immunosuppression.

Gene replacement therapy has the great potential of providing a definitive cure for patients affected with IEMs.

1.2 Disease candidates for liver-directed gene therapy

An ideal candidate for gene therapy is a disease in which there is an immediate and simple measure of efficacy, in which the only alternative therapy is liver transplantation, and a disorder that benefits from low expression levels of the defective enzyme. How much correction is needed for phenotypic correction ultimately depends on the magnitude of the metabolic flux through the biochemical pathway at the cellular and tissue levels. This is determined by the capacity of the biochemical pathway to be up-regulated by

overexpression of various rate-limiting and non rate-limiting steps and by the degree of cellular autonomy of the target enzyme (BRUNETTI-PIERRI and LEE 2005). The scale of metabolic correction has been determined indirectly by animal gene transfer studies (**Figure 1**). In general, disorders that are non-cell autonomous, such as hemophilia A and B or the mucopolysaccharidoses (MPS), require less transduction of the liver than cell autonomous defects such as the urea cycle disorders. This is partially due to the potential of cross-correction when secreted proteins in the MPS act distally either outside or inside the target cells. At the same time, the magnitude of metabolic flux is also critical in determining the percentage of liver transduction required for phenotypic correction. Diseases due to enzymatic deficiencies may generally require higher percentage of correction compared to diseases such as hemophilia (BRUNETTI-PIERRI and LEE 2005). Some of the candidate disorders for liver-directed gene therapy are listed in **Table 1**. Homozygous familial hypercholesterolemia (FH) is an attractive disorder but there is significant risk of atherosclerosis with LDL-receptor levels that are 50% of normal. It is likely that relatively low levels of expression would achieve some lowering of cholesterol in homozygous FH, and this might provide some measure of efficacy. However, much higher levels of expression are likely needed to obtain optimal benefit in homozygous FH. Glycogen storage disease (GSD-1a) also are likely required to obtain full phenotypic correction although a smaller percentage may be sufficient for correction of the hypoglycemia. Ornithine transcarbamylase (OTC) deficiency is an alternative that might be considered, particularly in males otherwise scheduled for liver transplantation.

However, measurement of efficacy in OTC deficiency is somewhat complicated since it depends on dietary intake of protein and other variables.

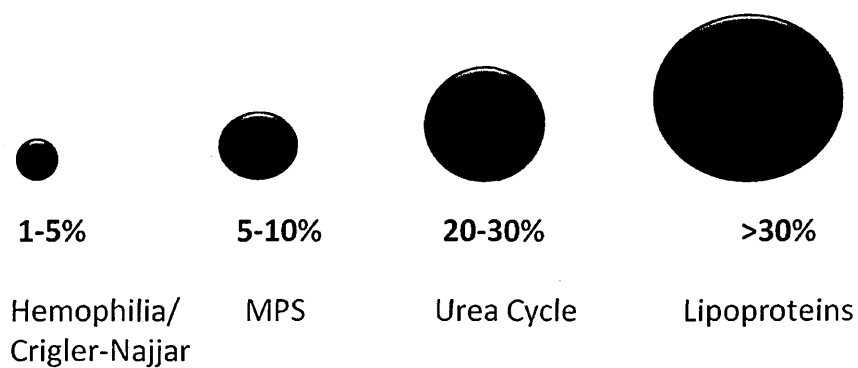


Figure 1. Percentage of hepatocytes expressing the normal gene required for phenotypic correction of different liver metabolic disorders (MPS=mucopolysaccharidoses) (BRUNETTI-PIERRI and LEE 2005).

Disorder	Patient enrollment	Alternative therapy	Low expression likely beneficial	Efficacy endpoint
Crigler-Najjar type I	Very rare	Liver transplant	Yes	Bilirubin levels
GSD-Ia	More feasible	Liver transplant	Yes	Glucose levels
Hemophilias (A and B)	More feasible	Protein replacement	Yes	Factor levels
FH (homozygous)	Very rare	Liver transplant	Likely need high expression	LDL levels
OTC deficiency	More feasible	Liver transplant	Partially	More difficult

Table 1. Candidate disorders for liver-directed gene therapy.

Crigler-Najjar syndrome (CN) type I is an excellent candidate disease for gene therapy for several reasons:

- it is a life-threatening disease requiring cumbersome treatment with phototherapy or liver transplantation (STRAUSS *et al.* 2006), and the risk/benefit ratio is favorable;
- the underlying defect is well characterized at the biochemical and molecular levels;
- there is evidence that low levels of gene expression will ameliorate the hyperbilirubinemia, as suggested by hepatocyte transplantation studies (CHOWDHURY *et al.* 1998) and because patients with CN type II have marked reduction but not total loss of enzyme activity, and their disease is much milder than type I;
- the outcomes of the experimental therapy can be easily monitored by measuring bilirubin in serum;
- it does not affect liver architecture;
- an animal model, the Gunn rat, is available to investigate experimental therapies (CHOWDHURY *et al.* 1993).

CN type I is also attractive for gene therapy because UGT1A1 can be produced from skeletal muscle other than liver, its natural production site, and still retain the ability to transform bilirubin into water-soluble derivatives (DANKO *et al.* 2004; JIA and DANKO 2005a).

The biggest negative of CN is the relative rarity of patients. The exact prevalence of CN is unknown but it is estimated to be as low as 1 case in 1 million live births. In some

populations (for example in from North Africa or the Amish and Mennonites) its prevalence is much higher.

2. INTRODUCTION

2.1 Crigler-Najjar Syndrome

In 1952, Crigler and Najjar first described a syndrome presenting with severe, chronic hyperbilirubinemia (CRIGLER and NAJJAR 1952). CN (MIM 21880) is an autosomal recessive disorder presenting with non-hemolytic unconjugated hyperbilirubinemia. The disease is due to mutations (deletions, insertions, missense, nonsense, and splicing mutations) in the bilirubin-uridinediphosphoglucuronate glucuronosyltransferase (*UGT1A1*) gene on chromosome 2 encoding the hepatic enzyme uridine diphosphate glucuronyltransferase (UDPGT) which catalyzes the glucuronidation of bilirubin. This reaction is an essential step in excretion of bilirubin into the bile.

Two clinical forms of this syndrome have been described (**Table 2**): the more severe CN type I is characterized by high levels of serum unconjugated bilirubin (greater than 340 μ M or 7-28 mg/dl, while the normal range is 0.2-1.0 mg/dl) owing to the nearly absence of UGT1A1 enzyme activity; and the milder CN type II with decreased UGT1A1 enzymatic activity that presents unconjugated serum bilirubin levels in the range of 60-340 μ M (or 3-20 mg/dl). Mutations in the promoter of the *UGT1A1* gene are responsible for Gilbert syndrome, a common benign condition with mild, unconjugated hyperbilirubinemia (serum bilirubin levels are of 60 μ M or below) that affects approximately 5% of the population (BOSMA *et al.* 1995).

Besides the levels of serum bilirubin, the difference between CN type I and II is based on clinical demonstration of efficacy of phenobarbital: in CN type I, the hyperbilirubinemia is not affected by the drug, whereas phenobarbital induces a rapid decline of serum bilirubin in CN type II.

	Crigler-Najjar Syndrome Type I	Crigler-Najjar Syndrome Type II	Gilbert Syndrome
Histology of the liver	Normal	Normal	Normal
Serum bilirubin concentration	20-50 mg/dl	<20 mg/dl	Usually < 3 mg/dl
Hepatic UGT activity	Absent	Markedly reduced	Reduced
Effect of phenobarbital on serum bilirubin	None	Reduction	Reduction
Mode of inheritance	Autosomal recessive	Autosomal recessive	Autosomal recessive
Prevalence	Rare	Rare	Common
Prognosis	Kernicterus, unless vigorously treated	Usually benign, kernicterus occurs rarely	Benign

Table 2. Differences between Crigler-Najjar syndrome type I and II and Gilbert syndrome.

The reduction of hyperbilirubinemia induced by phenobarbital depends upon generalized induction of liver enzymes including UDPGT.

Patients with CN type I have elevated serum bilirubin levels that are refractory to phenobarbital treatment. The increase of serum bilirubin can be life-threatening and to maintain serum bilirubin below the threshold resulting in brain damage, affected patients generally sleep and spend extended hours under the visible blue lights throughout childhood and adolescence (CREMER *et al.* 1958). Despite this therapy, they remain at risk of brain damage when intercurrent infections increase the production of bilirubin above levels that can be controlled by phototherapy (STRAUSS *et al.* 2006). In addition, the amount of time required under bilirubin lights tends to increase with age as there is a change in ratio of body surface area to body mass (CREMER *et al.* 1958). Thus, patients with CN type I are often advised to consider liver transplantation, most frequently in the range of 18-25 years of age.

2.2 Bilirubin

Bilirubin is the normal breakdown product of the protoporphyrin portion of the heme group of proteins such as hemoglobin, myoglobin, and cytochrome P-450. An individual with normal human metabolism generates 250-400 mg/day of bilirubin through the breakdown of these hemoproteins (IYANAGI *et al.* 1998). The first step in the degradation of the heme group to bilirubin is cleavage of the α -methene bridge of heme to form biliverdin (SAITO and ITANO 1982). This reaction is catalyzed by microsomal the heme oxygenase (HO) that requires O₂ and NADPH. Biliverdin is then reduced to bilirubin by the cytosolic biliverdin reductases which require NADH or NADPH (**Figure 2**).

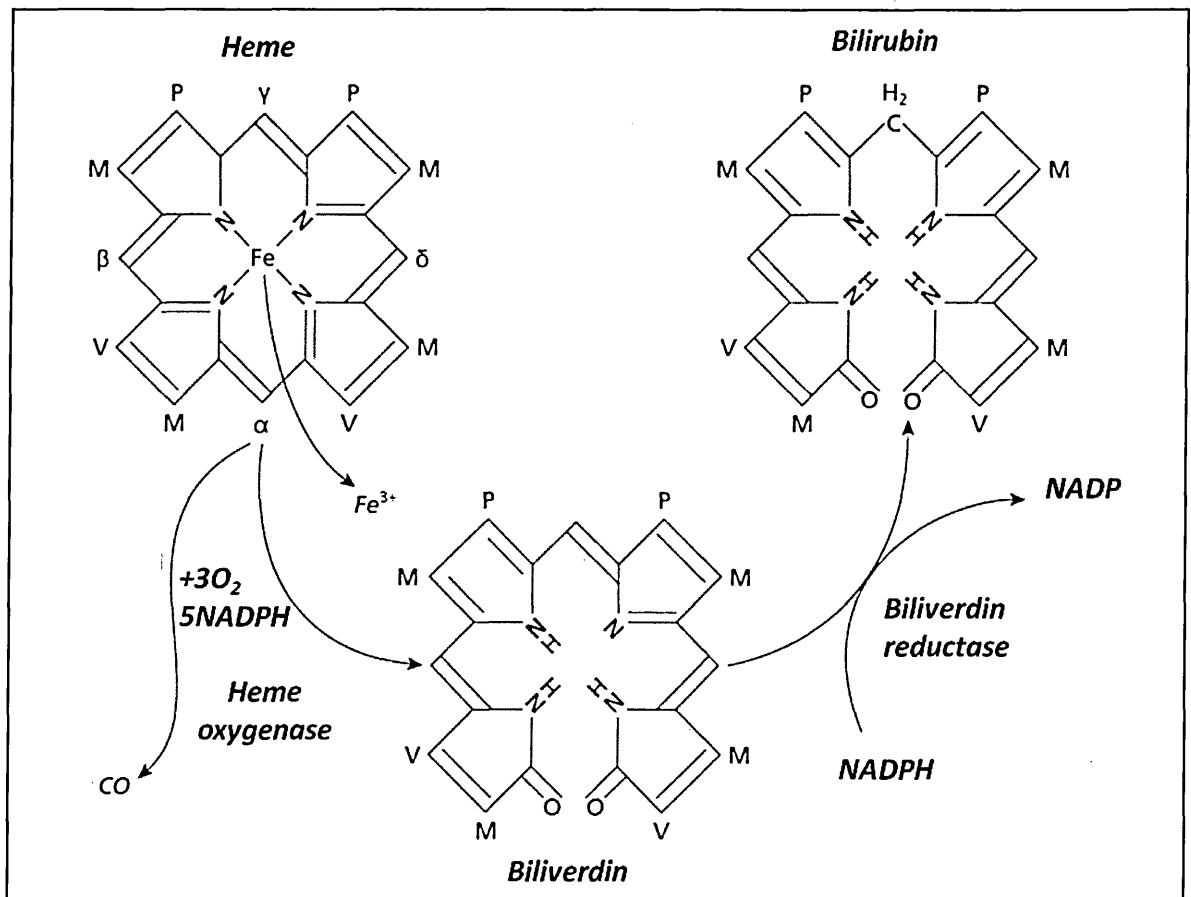


Figure 2. Enzyme-catalyzed degradation of heme. Heme degradation begins by heme oxygenase-catalysed oxidation of the α -bridge carbon of heme, which is converted to CO, leading to opening of the tetrapyrrole ring and release of the iron. The resulting biliverdin molecules are next reduced to bilirubin by cytosolic biliverdin reductase.

Bilirubin is an asymmetrically substituted tetrapyrrole dicarboxylic acid. The introduction of CH₂ at C-10 of the bilirubin molecule induces a conformational change by rotation of dipyrnone groups around the central CH₂ group. This allows intramolecular hydrogen bonding of the propionic acid carbonyl to the amino groups of the dipyrnone lactam and the pyrrole ring (NOGALES and LIGHTNER 1995) that explain several properties of bilirubin, such as its high lipid/water partition coefficient and its resistance to hepatobiliary excretion. Bilirubin requires conjugation with glucuronic acid for biliary excretion.

2.3 Bilirubin toxicity

The immaturity of most steps of bilirubin metabolism causes a mild, temporary retention of unconjugated bilirubin (UCB) in approximately 60% of healthy term neonates who show serum UCB levels of less than 170 μ M (10 mg/dL) within the first days of life (OSTROW *et al.* 1994). The hyperbilirubinemia is clinically evidenced by deposition of bilirubin in the sclera and in the skin that became yellow. The term ‘icterus’ from the Greek *ikteros* or “jaundice” from the French “jaune” that mean yellow, is used to describe this condition. The neonatal jaundice resolves spontaneously without sequelae within the first week of life in most cases (OSTROW *et al.* 2003). However, moderate hyperbilirubinemia, (200-300 μ M; 11.7 mg/dL) occurs in at least 16% of infants. The increased uptake of UCB into the central nervous system may cause transitory effects such as hypotonia, lethargy, anorexia, poor suckling, and development of bilirubin encephalopathy or bilirubin induced neuronal dysfunction (BIND) that is usually reversible.

In about 2% of infant with higher or prolonged hyperbilirubinemia (UCB \geq 13-26 mg/dL; 220-440 μ M) there is increased risk of permanent neurological complications

ranging from motor development delay, impaired cognitive functions, auditory dysfunctions to more severe motor, auditory and cognitive disorders, and death (SOORANI-LUNSING *et al.* 2001). Yellow discoloration of the hippocampus, basal ganglia and nuclei of cerebellum and brain stem found in infants with acute bilirubin encephalopathy is termed *kernicterus* that means "yellow kern," with kern indicating the most commonly afflicted region of the brain (i.e., the nuclear region). At serum unconjugated bilirubin concentrations over 20 mg/dL, newborn babies are at risk of *kernicterus*.

The treatment of neonatal jaundice is based on phototherapy that converts UCB into photoisomers that can be excreted in bile and urine. In newborns with severe jaundice, more aggressive treatments including exchange transfusion to physically remove UCB from the circulation are undertaken.

UCB is toxic to many cell types and it inhibits DNA synthesis (SCHIFF *et al.* 1985), ATPase activity of brain mitochondria (MUSTAFA *et al.* 1969), and uncouples oxidative phosphorylation. Which of these toxic effects is the cause of bilirubin encephalopathy remains unclear at this time. Because the toxic effects of bilirubin are abrogated by tight binding to albumin, cerebral toxicity is usually observed when there is a molar excess of bilirubin over albumin in plasma.

2.4 UCB glucuronidation

Unconjugated bilirubin circulates in plasma only bound tightly but reversibly to serum albumin, which prevents its excretion in urine, except during albuminuria. Albumin binding keeps bilirubin in solution and abrogates its toxic effects. Conjugated bilirubin is bound less tightly to albumin, and the unbound fraction is excreted in the urine.

At the sinusoidal surface of the hepatocyte (**Figure 3**), bilirubin dissociates from albumin and is taken up by the hepatocyte by facilitated diffusion that requires inorganic anions, such as Cl^- . The protein(s) involved in sinusoidal bilirubin uptake have not been identified yet. A member of the organic anion transport protein family, named OATP2 (also termed SLC21A6), has been proposed as the sinusoidal bilirubin transporter (CUI *et al.* 2001), but its importance in bilirubin transport has been questioned (WANG *et al.* 2003).

Once in the hepatocyte, bilirubin is bound to major cytosolic proteins, glutathione-*S*-transferases (GSTs, formerly designated as ligandin or Y-protein). GST is a small basic component making up to 6% of total cytosolic proteins of the liver. Bilirubin is a ligand for GSTs, but not a substrate for glutathione transferases. Binding to GSTs reduces the efflux of bilirubin from hepatocytes, thereby increasing its net uptake. GST binding inhibits non-specific diffusion of bilirubin into various subcellular compartments, thereby preventing specific organel toxicity, such as inhibition of mitochondrial respiration (KAMISAKA *et al.* 1975).

In the hepatocyte, the propionyl side chains of bilirubin are conjugated to form a diglucuronide. The reaction is catalized by UDPGT that utilizes uridine diphosphoglucuronate (UDPGA) as a glucuronate donor to bilirubin (**Figure 4**). The conversion of unconjugated bilirubin to bilirubin diglucuronide or monoglucuronide is critical for efficient biliary excretion of bilirubin.

In normal bile, the diglucuronide is the major form of excreted bilirubin and only small amounts of monoglucuronide or other glycosidic adducts are present. Unconjugated bilirubin accounts for less than 10%. In addition to glucuronides, small amount of glucosyl, xylosyl and mixed conjugates of bilirubin are found in human bile.

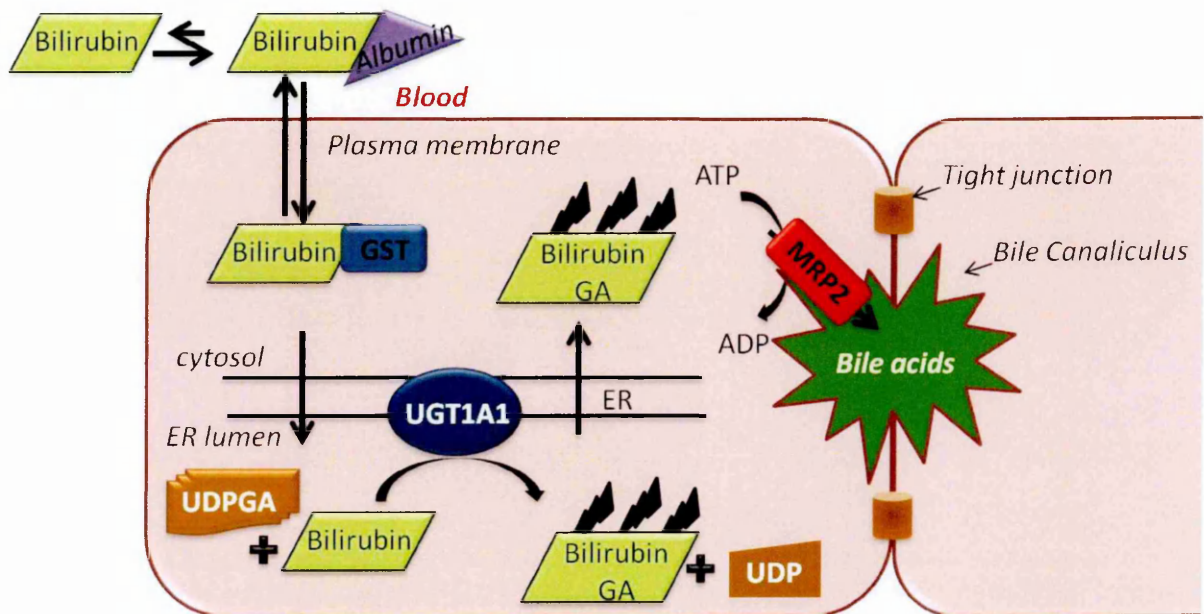


Figure 3. Hepatocyte bilirubin metabolism. Bilirubin is transported from sites of production to hepatic sinusoids bound to albumin. At sinusoidal surface of hepatocytes, bilirubin dissociates from albumin and enters hepatocytes by facilitated diffusion. Binding to cytosolic GST increases net uptake of bilirubin by inhibiting its efflux. Bilirubin is converted to mono- and diglucuronide by the UGT1A1, which catalyses the transfer of the glucuronic acid moiety from UDPGA to bilirubin. Bilirubin glucuronides (Bilirubin GA) are actively transported into bile against a concentration gradient by the ATP-utilizing pump MRP2.

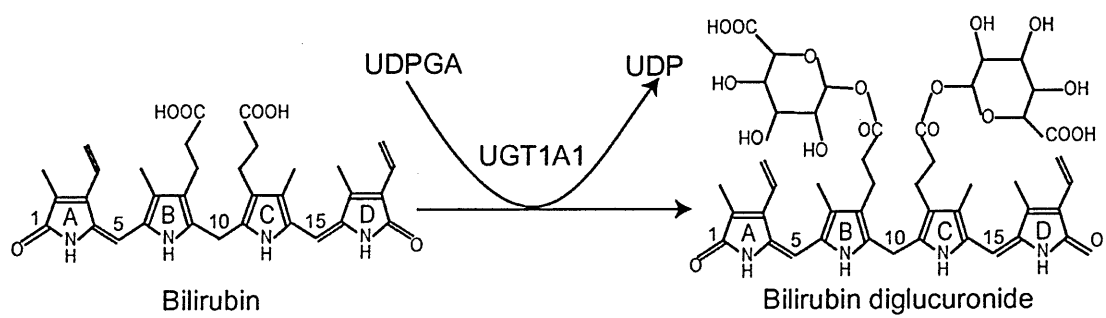


Figure 4. Bilirubin glucuronidation reaction catalyzed by UGT1A1.

Bilirubin diglucuronide is much more water-soluble than free bilirubin, and thus the transferase facilitates excretion of bilirubin into the bile.

Conjugated bilirubin undergoes unidirectional transport into the bile against a concentration gradient, so that bilirubin concentration in the bile can be up to 150-fold higher than hepatocyte concentration. The electrochemical gradient of -35 mV, generated by the sodium pump, helps the canalicular transport but it is not sufficient by itself to generate such a large concentration gradient. The energy for transport of bilirubin is derived from adenosine triphosphate (ATP) hydrolysis by the canalicular ATP-binding cassette protein, the MDR-related protein 2 (MRP2). MRP2 pumps glutathione-, glucuronic acid- or sulphate-conjugated compounds across the canalicular membrane (ISHIKAWA *et al.* 1990; NISHIDA *et al.* 1992). Canalicular transport of organic anions is unidirectional from the cytoplasm of the hepatocyte to the bile.

2.5 The UGT family

Uridine diphosphoglucuronate glucuronosyl transferases (UGTs) are a superfamily of enzymes concentrated in the endoplasmic reticulum (ER) and nuclear envelope of several cell types and catalyze glucuronidation of several molecules. These enzymes play an important role in clearance of environmental toxins, endogenous metabolites, and drugs. They catalyze transfer of glucuronic acid moiety of UDPGA to the aglycone substrates, forming polar and usually less bioreactive products. Bilirubin glucuronidation is catalyzed predominantly by a single UGT isoform, the UGT1A1 (BOSMA *et al.* 1994).

Based on identity in amino acid sequence, UGTs are divided into two families, UGT1 and UGT2 (BURCHELL *et al.* 1991). Members of the UGT1 gene family include

bilirubin- and phenol-metabolizing isoforms. The UGT1 subfamily of UGT proteins catalyzes the glucuronidation of donor substrate (UDPGA) to several acceptor substrates. The diversity of UGT1 proteins is determined by the genomic organization of genes encoding for them. The rat and human *UGT1* clusters have been well characterized (EMI *et al.* 1995). Nine isoforms within the UGT1A subfamily are expressed from a series of exons clustered in a unique manner on chromosome 2 at the 2q37 region. Four consecutive exons (exons 2–5) located at the 3' end of the UGT1A locus are used by nine different mRNAs (**Figure 5**). These encode the identical carboxy-terminal domains of these UGT isoforms, which contain the UDPGA binding site. Upstream of these four common region exons is a series of unique exons, each preceded by a separate promoter. Only one of these exons is used in a specific UGT mRNA. The unique exon encodes the variable N-terminal domain of the nine different UGT isoforms dictate aglycone specificity to each individual isoforms. Depending on the promoter used, transcripts of various lengths are generated. In all cases, the unique exon, located at the 5' end of the transcript, is spliced to exon 2, and the intervening sequence is spliced out. The genes are named according to the unique first exon. Thus, UGT1A1 utilizes the unique exon 1A1, UGT1A6 utilizes exon 1A6, etc. The presence of a separate promoter upstream from each unique exon permits differential regulation of individual UGT isoforms during development and in response to inducing agents.

UGT1A1 is expressed after birth and is induced by phenobarbital and clofibrate (CHOWDHURY *et al.* 1983). Delayed expression of UGT1A1 is a major cause of neonatal hyperbilirubinaemia. In humans, the expression of UGT1A1 is limited to hepatocytes and, to a lesser extent, to the proximal small intestine.

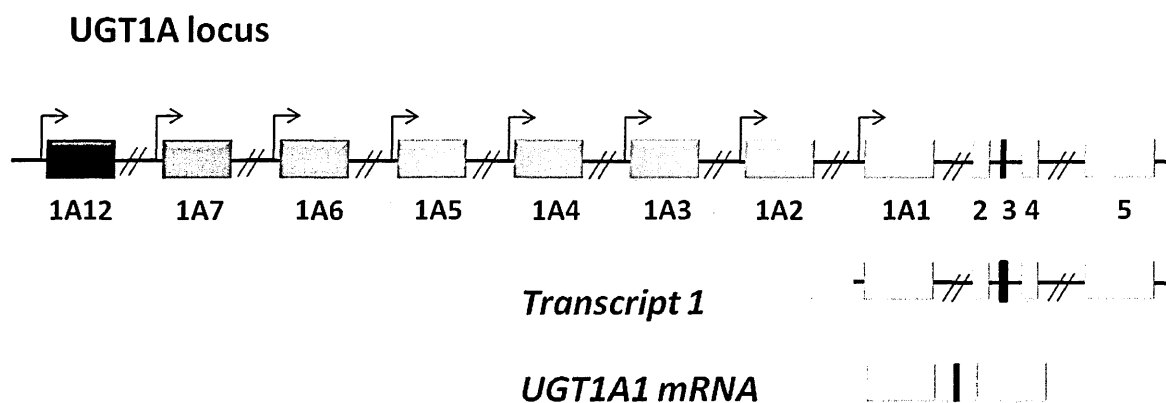


Figure 5. Organization of the UGT1 gene complex locus. This locus contains at least eight exons 1, which can be spliced to common exons 2 through 5. The first exons are named 1A1, 1A2, 1A3, etc, and accordingly their gene products are named UGT1A1, UGT1A2, UGT1A3, etc.

The DNA sequence of UGTs contains an amino-terminal signal peptide, which is cleaved during synthesis of the polypeptide chain (IYANAGI *et al.* 1986), and a stretch of 17 hydrophobic amino acids near the carboxy terminus, that anchors the protein to the lipid bilayer (MACKENZIE 1986). The stretch of 20-30 amino acids at the carboxy terminus of the UGT contains several basic residues, which are responsible for retaining the UGT in the ER (KINOSAKI *et al.* 1993).

Members of the UGT2 family include steroid-metabolizing isoforms. Comparison of the amino acid sequences of the proteins encoded by the UGT2 gene family reveals differences in amino acid sequence throughout the length of the protein. The carboxy-terminal halves, however, are highly conserved, which may provide the binding site for the common co-substrate, UDPGA (IYANAGI *et al.* 1986; MACKENZIE 1986).

2.6 Molecular defect in CN type I syndrome

Mutations in the *UGT1A1* gene are responsible for both type I and type II CN and were first described in 1992 (BOSMA *et al.* 1992a; BOSMA *et al.* 1992b; BOSMA *et al.* 1994). The genetic lesions can be deletions, insertions, missense mutations, or premature stop codons (KADAKOL *et al.* 2000). The severity of the functional deficiency of UGT1A1 is determined by the nature of the genetic lesion. CN type I results from premature truncation or critical amino acid residue substitution whereas CN type II is caused by substitution of single amino acid residues that reduce but not abolish the enzyme activity. The TA insertion within a dinucleotide repeat in the promoter region of *UGT1A1* gene is responsible for Gilbert syndrome. The normal TATAA element sequence is A(TA)₆TAA, whereas in Gilbert syndrome the sequence is A(TA)₇TAA. The variant TATAA element reduces the expression of the structurally normal enzyme (BOSMA *et al.* 1995).

2.7 Current Therapies for Crigler-Najjar Syndrome

Treatment of CN is mainly based on inhibition of bilirubin production (heme oxygenase inhibitors), stimulation of bilirubin metabolism (phenobarbital), increase clearance of bilirubin (phototherapy), and removal of excessive bilirubin by plasma exchange transfusion in the severe cases.

The main therapy for CN type I is phototherapy. The visible blue lights (420-460 nm) transform the bilirubin in its photoisomers (referred also as photobilirubin and lumirubin) which are water soluble and therefore can be excreted in the urine. Phototherapy has to be carried out for at least 10-12 hours using lamps that emit light with appropriate wavelengths. The amount of time required under bilirubin lights tends to increase with age as there is a change in ratio of body surface area to body mass (CREMER *et al.* 1958). Plasmapheresis is the most efficient method for rapidly reducing serum bilirubin concentration during acute crisis. Given the increased risks of irreversible brain damage and death, patients with CN type I are often advised to consider liver transplantation, most frequently in the range of 18-25 years of age. Orthotopic or auxiliary liver transplantation rapidly normalizes serum bilirubin levels. Currently, liver transplantation is considered the only definitive treatment for CN type I. However, liver transplantation is far from being an ideal therapy because is limited by the number of donor organs available, transplant mortality and morbidity, and life-long immunosuppression.

Hepatocyte transplantation (transplantation of hepatic cells, i.e. hepatocytes, rather than whole liver) is an alternative to liver transplantation. Matas et al. have demonstrated that infusions of immunosuppressed Gunn rats with normal hepatocytes via the portal vein result in reduction of plasma bilirubin concentration, ameliorating clinical symptoms of the disease (MATAS *et al.* 1976). Two decades later, Fox et al. treated a patient with CN type I

with hepatocyte transplantation (FOX *et al.* 1998). The 11-year-old girl with CN was transplanted with 7.5 billion isolated allogeneic primary human hepatocytes by infusion through a percutaneously placed portal venous catheter (CHOWDHURY *et al.* 1998; FOX *et al.* 1998). Tacrolimus was used for prevention of allograft rejection. The procedure resulted in excretion of bilirubin glucuronides in bile, and an approximately 50% reduction of serum bilirubin concentration over the course of several months. Unfortunately, the amount of bilirubin-UGT enzyme activity derived from the transplanted cells was not sufficient to eliminate the patient need for organ transplantation.

Given the limitations of currently available treatments, it would be desirable to develop more effective treatments for this severe disease, and gene therapy has a great potential to provide a definitive cure for this disorder (MIRANDA and BOSMA 2009).

2.8 Animal models of Crigler-Najjar syndrome type I

The Gunn rat is a mutant strain of Wistar rats that lacks the UDPGT activity toward bilirubin and develops unconjugated hyperbilirubinemia (**Figure 6A**). In this animal model the genetic lesion closely parallel those in CN type I (CHOWDHURY *et al.* 1993) and the brain lesions are similar to those in humans, with cell loss and gliosis most prominent in the auditory nuclei of the brainstem, the cerebellum, the hippocampus and the basal ganglia (CHOWDHURY *et al.* 1993). This strain carries a spontaneous single-base-pair deletion of the *UGT1A1* gene. The deletion of a single base pairs in the UGT1 common exon 4 produces a stop codon that remove the 115 amino acids from the carboxy terminus of the protein (approximately 13 KDa) responsible for the binding on the ER (**Figure 6B**). The mutant protein is unstable and rapidly degraded (BURCHELL *et al.* 1991; CHOWDHURY *et al.* 1993;

ELAWADY *et al.* 1990; IYANAGI *et al.* 1989). Pulse-chase experiments using primary cell cultures showed that the truncated UGT1A1 protein of Gunn rat is synthesized similarly to wild-type protein. However, the truncated UGT1A1 protein is degraded more rapidly with a half-life of about 50 minutes, whereas the wild-type UGT1A1 protein has a much longer half-life of about 10 hours (**Figure 6C, D**) (EMI *et al.* 2002). In Gunn rat the mutation in the exon 4 (common) causes the simultaneous deficiencies of all UGT1 isoforms (EMI *et al.* 1995).

The homozygous *jj* Gunn rats have hyperbilirubinemia (CHOWDHURY *et al.* 1991; IYANAGI *et al.* 1989) while the non jaundiced heterozygous *Jj* rats have normal serum bilirubin (SCHMID *et al.* 1958). Total serum bilirubin (TSB) levels in Gunn rats range from 3 mg/dL (50 μ M) to 20 mg/dL (340 μ M) depending on the strain and the diet (normal TSB levels in rats are 0.01-0.05 mg/dL). The bile of homozygous Gunn rats contains trace amounts of bilirubin mono-conjugates (BLANCKAERT *et al.* 1977; WISHART 1978).

For more than 60 years, Gunn rats have been intensively used both to understand the disease pathogenesis and to develop therapeutic approaches (CHOWDHURY *et al.* 1993). The disease in Gunn rats is relatively milder compared to humans affected by CN type I.

The Gunn rats survive and reach adulthood without any treatment, and they may only show cerebellar hypoplasia, hearing impairment, and minor behavioral defects (CHOWDHURY *et al.* 1993) which disappear after a short period of phototherapy. Despite these important differences with the human disease, this animal model has been highly instrumental to better understand the biology of CN and to validate a wide variety of therapeutic approaches.

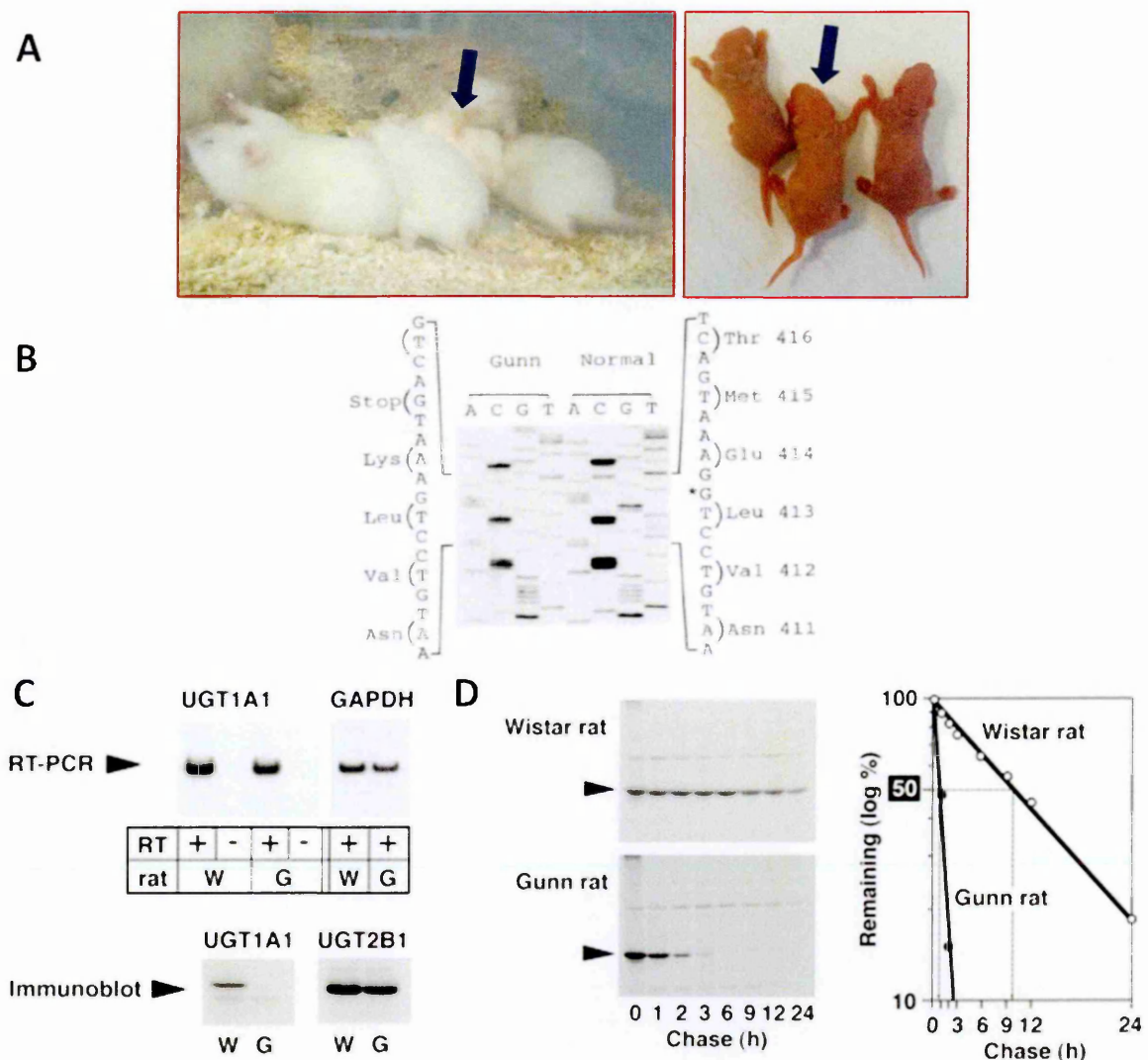


Figure 6. The Gunn rat. **A.** Gunn rats at 3 and 20 days post-natal age. The *jj* hyperbilirubinemic rats (arrows) exhibit yellow discoloration of the skin (see ears and tail) and coat, whereas heterozygous (*Jj*) animals show normal skin and coat color. **B.** Nucleotide sequence of UDPGT cDNAs from normal Wistar and homozygous Gunn rats. The deletion is marked with an asterisk. **C, D.** Absence of UGT1A1 protein in Gunn rat hepatocytes (Emi et al., 2002). The UGT1A1 mRNA is present in Gunn rats even if at lower amounts but the protein is undetectable. The mutation in Gunn rats results in accelerated degradation of truncated UGT1A1 protein.

Nguyen et al. in 2008 generated a mouse model in which the coding region of exon 4 has been interrupted resulting in levels of UCB that are comparable to those observed in patients. The absence of functional UGT1A protein predisposes neonatal mice to fatal consequences related to the high levels of unconjugated hyperbilirubinemia (FUJIWARA *et al.* 2010; NGUYEN *et al.* 2008). Despite having an identical mutation, the phenotype observed in the mice is much more severe than the Gunn rats. The reasons for such striking differences are not known. Another mouse model carrying a 1-base deletion in exon 4 of the Ugt1 has also been generated and it shows a similar phenotype (BORTOLUSSI *et al.* 2012). The hyperbilirubinemia in these mice was corrected by intraperitoneal injection of an AAV9 vector expressing the hUGT1A1 under the control of the ubiquitous CMV promoter.

2.9 Gene therapy for Crigler-Najjar syndrome type I

Over the last two decades, *ex vivo* and *in vivo* gene therapy using viral and non-viral vectors has been used to correct hyperbilirubinemia in the animal models of CN (BELLODI-PRIVATO *et al.* 2005; KREN *et al.* 1999; MIRANDA and BOSMA 2009; NGUYEN *et al.* 2007; SAUTER *et al.* 2000; SCHMITT *et al.* 2010b; SEPPEN *et al.* 2003; TAKAHASHI *et al.* 1996; TOIETTA *et al.* 2005).

An important advantage of *ex-vivo* gene therapy is that triggering of innate immune responses is avoided (MIRANDA and BOSMA 2009). In addition, it prevents systemic dissemination of the vector to multiple tissues that are not relevant for disease correction. *Ex-vivo* gene therapy has been used successfully to correct deficiencies in hematopoietic stem cells (QASIM *et al.* 2004). The success of this approach is dependent on efficient

repopulation of the hematopoietic system by the corrected cells due to their growth advantage over uncorrected cells. In hepatic disorders that result in hepatocyte injury, efficient repopulation of the liver can also be achieved (DE VREE *et al.* 2000; OVERTURF *et al.* 1998). However, in most inherited inborn errors of liver metabolism, including CN, corrected hepatocytes do not have a selective growth advantage and thus the efficacy of transplanted genetically modified cells is limited.

Correction of CN type I by gene therapy has been the goal of several *in vivo* studies using different vector systems including Retrovirus (RV), Lentivirus (LV), Adenovirus (Ad), Adeno-Associated Virus (AAV), and non-viral vectors. RV expressing UGT1A1 injected in newborns (BELLODI-PRIVATO *et al.* 2005) or in conjunction with partial hepatectomy (TADA *et al.* 1998) has achieved long-term correction of hyperbilirubinemia in the Gunn rats. LV vectors can also transduce non-proliferating cells and in the Gunn rats they resulted in stable reduction of bilirubin levels to near normal levels for over 1 year after treatment (VAN DER WEGEN *et al.* 2006). Nguyen *et al.* also reported that delivery of murine leukemia virus (MLV)-based retroviral or lentiviral vectors to newborn Gunn rats resulted in sustained reduction of hyperbilirubinemia for at least 40 weeks (BELLODI-PRIVATO *et al.* 2005; NGUYEN *et al.* 2005; SCHMITT *et al.* 2010a; SCHMITT *et al.* 2010b).

Impressive lifelong correction of hyperbilirubinemia has been reported in the Gunn rats following a single intravenous injection of helper-dependent adenoviral vector (HDAd) encoding UGT1A1 with negligible chronic toxicity (TOIETTA *et al.* 2005). Recently, it has been demonstrated that an improved expression cassette combined with a more efficient method of vector delivery (hydrodynamic injection) permits correction of hyperbilirubinemia in the Gunn rat using clinically relevant low HDAd doses (DIMMOCK *et al.* 2011). As a part of a research project, not included in this thesis work, I have also

recently demonstrated that direct intrahepatic injections, by either surgical exposure of the liver or by ultrasound-guided percutaneous injections, of HDAd vector expressing UGT1A1 results in improved phenotypic correction at low clinically relevant doses compared to systemic intravenous injections (Pastore et al, submitted). In addition, a reduction of hyperbilirubinemia has also been reported following hydrodynamic injection of pDNA (JIA and DANKO 2005b).

Among the different vectors used so far, AAV vectors have emerged as the most promising. A previous study has investigated different serotypes in Gunn rats and AAV1 was previously found to be the most efficient for correction of the hyperbilirubinemia. It has to be noted that in that study a large hepatic macroscopic lipid lesions of unclear etiology were found in AAV-treated animals (SEPPEN *et al.* 2006).

Glucuronidation of bilirubin can occur in tissues other than liver and still result in reduction of hyperbilirubinemia. For example Seppen et al. (SEPPEN *et al.* 1997) used RV vectors to transfer the human UGT1A1 gene into autologous ear fibroblasts of the Gunn rats that were then injected into the peritoneum. This therapeutic strategy resulted in complete correction of serum bilirubin levels. However, the animals developed cancer because injected fibroblasts acquired tumorigenic properties due to prolonged culturing *in vitro*. Nevertheless, this study demonstrates that expression of UGT1A1 in an ectopic tissue corrects the hyperbilirubinemia in the animal model of CN.

3. SPECIFIC AIMS

The overall goal of my thesis was to generate preclinical data to support clinical applications of a gene-based strategy for the therapy of CN type I patients. Towards this goal, I focused on two main approaches one based on muscle gene transfer and another on liver-directed gene therapy.

Although correction of the deficient enzymatic activity in the affected organ, i.e. the liver, would be the most straightforward, expression within an ectopic tissue, different from the natural production site, is an attractive option for clearance of toxic metabolites from the circulation. Interestingly, transplantation of small-bowel and kidney, which also express UDPGT, were effective at reducing the hyperbilirubinemia in Gunn rats, thus suggesting that ectopic expression of the enzyme is sufficient for metabolic correction of the disease (KOKUDO *et al.* 1999).

Muscle has been the preferred target tissue for gene transfer because of its simple access by intramuscular injections and safety. AAV vectors are ideal candidates for muscle-directed gene therapy approaches and they have shown encouraging results in various preclinical disease models. Moreover, those studies have led to multiple human clinical trials (BRANTLY *et al.* 2009; MANNO *et al.* 2003; STROES *et al.* 2008) and to the development of Glybera (alipogene tiparvovec), an AAV vector for muscle gene therapy of lipoprotein lipase deficiency that has been the first gene-therapy medicine recommended for authorization in the European Union (GAUDET *et al.* 2013; YLA-HERTTUALA 2012).

Previous studies have shown reduction of hyperbilirubinemia in the Gunn rat by plasmid DNA delivery into skeletal muscle via limb perfusion (DANKO *et al.* 2004; JIA and DANKO 2005a). In those studies, injections of pDNA expressing human *UGT1A1* under the

CMV promoter resulted in excretion of bilirubin glucuronides in bile and short term reduction of serum bilirubin lasting for 2 to 4 weeks. Loss of correction was associated with a decrease in UDPGT protein in muscle, while pDNA and transcript were detectable up to 4 weeks after gene delivery. Longer correction required repeated pDNA delivery achieved by a relatively invasive procedure and immunosuppression (DANKO *et al.* 2004; JIA and DANKO 2005a).

The **specific aim 1** of my thesis was to investigate the safety and efficacy of muscle-directed gene therapy of CN type I using AAV vectors.

Although attractive for its safety, AAV mediated muscle-directed gene therapy of CN type I did not result in complete correction of the hyperbilirubinemia in the Gunn rats. Encouraged by the promising results of the hemophilia B clinical trial (NATHWANI *et al.* 2011b), I next investigated systemic intravenous injections for liver-directed gene therapy of CN type I by AAV vectors. This approach has the potential to completely correct the hyperbilirubinemia in CN type I.

Liver-directed gene therapy with AAV vectors has been difficult to accomplish. In the first hemophilia B gene therapy trial, the administration of AAV2 encoding the human Factor IX (FIX) resulted in transaminase elevation and a decline of FIX levels due to a specific immune-mediated destruction of the transduced cells (MANNO *et al.* 2006). The mechanisms involved in this immune reaction are not completely understood but it is clear that CD8⁺ T-cells against AAV capsid proteins are involved in the clearance of the transduced hepatocytes (HASBROUCK and HIGH 2008). Based on the discovery that other AAV serotypes displayed higher tropism for liver compared to AAV2, another clinical trial for liver-directed gene therapy was started in hemophilia B patients using serotype 8 AAV vector injected through a peripheral vein. In this trial no immune reaction was observed at

the lower doses while two participants who received the higher dose of vector developed a transient, asymptomatic elevation of serum aminotransferase levels which was associated with the detection of AAV8-capsid-specific T cells in peripheral blood. Both these participants received a short course of glucocorticoid therapy which rapidly normalized aminotransferase levels and maintained FIX in the therapeutic range. In all patients, AAV8 administration resulted in therapeutic FIX levels at 2-11% of normal levels, four of the six discontinued FIX prophylaxis and remained free of spontaneous hemorrhage, and in the other two the interval between prophylactic clotting factor infusions was increased (NATHWANI *et al.* 2011b).

The **specific aim 2** of my thesis work was to optimize the gene therapy vector for CN type I and to generate preclinical data using AAV2/8 in the Gunn rats to support a gene therapy clinical trial.

4. MATERIALS AND METHODS

4.1 Construction and production of AAV vectors

The rat UGT1A1 (rUGT1A1) coding sequence was obtained from Wistar rat liver mRNA and cloned into the pAAV2.1-MCK-eGFP plasmid (TESSITORE *et al.* 2008) by replacement of the enhanced green fluorescent protein (eGFP) sequence (**Figure 7A**). The cloned rUGT1A1 sequence was entirely verified by direct DNA sequencing.

The human UGT1A1 (hUGT1A1) coding sequence was cloned downstream the thyroxine binding globulin (TBG) promoter into the pAAV2.1-TBG-eGFP plasmid by replacing the eGFP sequence (**Figure 7B**). The pAAV2.1-TBG-hUGT1A1 contains the simian virus 40 (SV40) intron between the promoter and the cDNA and it also includes the Woodchuck Hepatitis Post-Transcriptional Regulatory Element (WPRE) that stabilizes the mRNA levels. The hUGT1A1 coding sequence was optimized (cohUGT1A1) for the expression in *Homo sapiens* (GeneArt). The codon usage was adapted to the codon bias of *Homo sapiens* genes. In addition, regions of very high (> 80 %) or very low (< 30 %) GC content have been avoided where possible. During the optimization process the following cis-acting sequence motifs were avoided where applicable: internal TATA-boxes and ribosomal entry sites; AT-rich or GC-rich sequence stretches; RNA instability motifs; repeat sequences and RNA secondary structures; (cryptic) splice donor and acceptor sites in higher eukaryotes. Codon optimized UGT1A1 was cloned into pAAV2.1-TBG-eGFP plasmid by replacing the eGFP sequence (**Figure 7B**).

A fragment containing the LP1 enhancer/promoter [a liver specific promoter containing the human apolipoprotein hepatic control region (HCR) and the human alpha-1-antitrypsin (hAAT) gene promoter including the 5' untranslated region] and the SV40

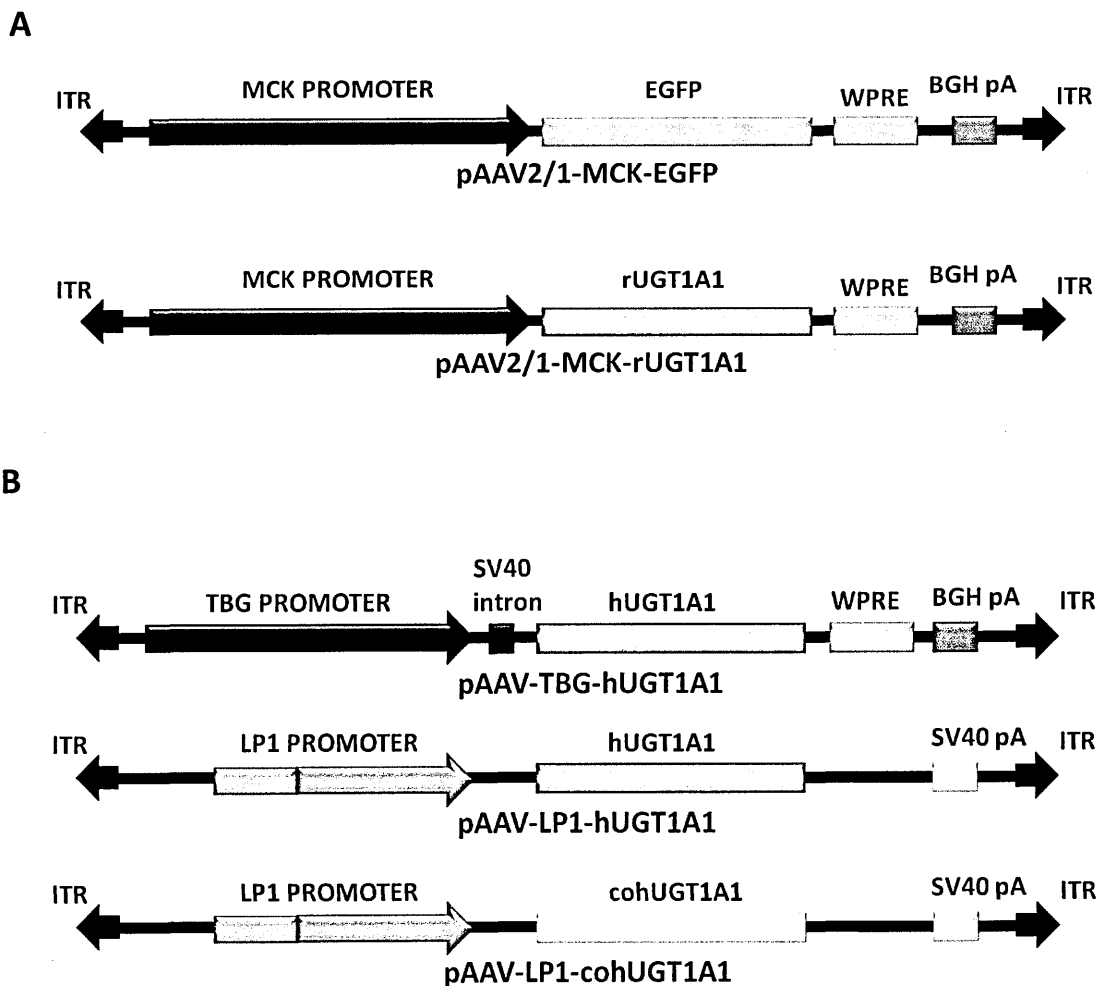


Figure 7. A. Muscle-specific AAV vectors. MCK, muscle specific creatin kinase promoter; eGFP, enhanced green fluorescent protein; rUGT1A1, rat uridine diphospho-glucuronosyl transferase 1A1 cDNA; WPRE, woodchuck hepatitis post-transcriptional regulatory element; BGHpA, bovine growth hormone poly-adenylation signal; ITR, inverted terminal repeats. **B. Liver-specific AAV vector genomes.** TBG, thyroxine binding globulin promoter; LP1, liver specific enhancer promoter; SV40 intron, simian virus 40 intron; hUGT1A1, human uridine diphospho-glucuronosyl transferase 1A1 cDNA; cohUGT1A1, codon optimized hUGT1A1; SV40 pA, simian virus 40 late polyadenylation signal, ITR, inverted terminal repeats. Not drawn to scale.

poly-A sequence was amplified using the scAAV-LP1-hFIX plasmid as template (kindly provided by Dr. Nathwani) after elimination of FIX cDNA and SV40 intron and cloned into the pAAV2.1-TBG-eGFP plasmid by replacing the TBG-eGFP-WPRE-BGH poly-A sequence to generate the pAAV2.1-LP1-SV40pA plasmid. The hUGT1A1 and the cohUGT1A1 coding regions were cloned into the pAAV2.1-LP1-SV40pA plasmid (**Figure 7B**).

The AAV vectors were produced and characterized by the Telethon Institute of Genetics and Medicine Vector Core (Naples, Italy). The pAAV2.1-MCK-rUGT1A1, pAAV2.1-MCK-EGFP, pAAV2.1-TBG-hUGT1A1, pAAV2.1-LP1-hUGT1A1, and pAAV2.1-LP1-cohUGT1A1 plasmids were triple-transfected in subconfluent 293 cells along with pAd-Helper and pack2/1 packaging plasmids as described previously (XIAO *et al.* 1999). Recombinant vectors were purified by two rounds of CsCl gradient centrifugation, as described previously (XIAO *et al.* 1999). Vector titers, expressed as genome copies per milliliter (GC/ml), were measured by both PCR quantification (TaqMan; PerkinElmer, Life and Analytical Sciences, Waltham, MA) and dot-blot analysis. For the comparison of AAV vectors with different expression cassettes for liver-directed gene therapy, the vector titer was calculated using serial dilutions of the plasmid AAV-TBG-hUGT1A1 and AAV specific primers (ITR F: 5'-GGAACCCCTAGTGATGGAGTT-3' and ITR R: 5'-CGGCCTCAGTGAGCGA-3'); the results were analyzed with LightCycler software version 3.5 (Roche).

4.2 Animal experiments

Animal procedures were performed in accordance with the regulations of the Italian Ministry of Health. Breeding pairs of Gunn rats were obtained from the Rat Resource and

Research Center (RRRC, Columbia, MO) and a colony of Gunn rats was established at the Institute of Genetics and Biophysics-Telethon Institute of Genetics and Medicine (IGB-TIGEM) animal facility (Naples, Italy).

For intramuscular (IM) injections, three injections (each of 30 μ l each) were performed in the gastrocnemius of 4-to-6 week-old male Gunn rats (75–150 g), for a total vector dose of 3×10^{12} genome copies (GC)/kg of AAV2/1-MCK-eGFP (n = 3), AAV2/1-MCK-rUGT1A1 (n = 9), or saline (n = 5), using a 100- μ l Hamilton syringe. Following vector injections, blood samples were collected by retro-orbital venipuncture at 3 weeks post-injection and each month thereafter. Bile was collected through a 26-gauge angiocatheter (Delta Med, Milan, Italy) inserted into the bile duct over 15-minute periods, protected from light, frozen, and store at -80°C until analyses. Random urine spots were collected for measurement of alkali-labile bilirubin concentrations. Urinary creatinine was measured by colorimetric assay based on the Jaffe method (CLARKE 1961). Creatine phosphokinase (CPK) was measured in serum samples in the first week and 3, 15, 24, and 52 weeks post-injection (Gentaur, Milan, Italy). To determine UGT1A1 activity, muscle and liver tissues were collected at 4 and 12 months post-injection. Tissues for real-time PCR were harvested from Gunn rats injected intramuscularly at 1, 4, and 12 months after injection.

For intravenous (IV) injections, AAV vectors or saline were injected in the tail vein of 4-to-6 week-old female Gunn rats (65-130 g) using a 26G needle. Following injections, blood samples were collected by retro-orbital venipuncture at 2 weeks post-injection and each month thereafter. Tissues for real-time PCR and western blot were harvested at 3 months post-injection.

4.3 Bilirubin determinations

Blood was centrifuged at 4000xg for 20 minutes and 8000xg for 10 minutes. Serum was used for colorimetric measurement of total bilirubin by a diazo-based assay (Gentaur, Belgium). The average serum bilirubin measured in 40 wild-type 3-4 week old Wistar rats was 0.91 - 0.51 mg/dl.

Biliary, serum, and urinary unconjugated bilirubin and alkali-labile pigment concentrations were determined by high-performance liquid chromatography (HPLC) as previously described (ZELENKA *et al.* 2008). Unconjugated bilirubin was measured before and after the addition of 1M NaOH to the sample for 10 minutes, and the concentration of alkali-labile bilirubin pigments, expressed as bilirubin equivalents, was calculated from the difference between the two measurements. Qualitative analysis of bilirubin pigments in bile was performed by direct HPLC of undiluted bile by the McDonagh method (TOIETTA *et al.* 2005) by our collaborator Dr. Antony McDonagh from University of California San Francisco (UCSF). Bilirubin determination in bile was performed by the collaborators Jana Vanikova and Libor Vitek, from Institute of Clinical Biochemistry and Laboratory Medicine, Charles University in Prague (Czech Republic).

4.4 Western blot analysis, enzyme assay, and qPCR on tissues

Muscle and liver samples were homogenized in 0.5 ml of phosphate-buffered saline (pH 7.4), using a TissueLyser homogenizer (Qiagen, Milan, Italy). The tissue homogenate was mixed with 4 ml of microsome buffer (2.62 mM KH_2PO_4 , 1.38 mM K_2HPO_4 , 2% glycerol, and 0.5 mM dithiothreitol) and first centrifuged at 12,000xg for 20 minutes at 4°C. The supernatant was then re-centrifuged at 105,000xg for 60 minutes at 4°C. The pellet was resuspended in microsome buffer and the protein concentration was determined

by the Bradford method. Microsomal extracts were characterized by detection of a calnexin band by Western blotting using anti-calnexin antibody (Assay Designs, Ann Arbor, MI). These extracts were used to measure UGT1A1 activity. Approximately 10-20 µg of microsomal proteins from Wistar and Gunn rat livers and muscles were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto PVDF membrane. Goat anti-rat UGT1A1 antiserum (diluted 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) with rabbit anti-goat IgG (diluted 1:3000; BioVision, Mountain View, CA) were used for immunodetection. Membranes were developed with an enhanced chemiluminescence kit (Thermo Scientific, Milan, Italy) and detected with Chemi- Doc (Bio-Rad, Hercules, CA).

UGT1A1 enzyme activity in muscle microsomes was measured according to a previously published assay using bilirubin as substrate (HEIRWEGH *et al.* 1972).

Total DNA was extracted from tissue samples using phenol-chloroform extraction and quantitated by absorbance at 260 nm.

Total RNA was extracted from livers of IV injected animals immediately placed in TRIzol reagent (Invitrogen, Monza, Italy) using an RNeasy kit (Qiagen, Italy). RNA was reverse transcribed using a first-strand complementary deoxyribonucleic acid kit with random primers according to the manufacturer's protocol (Applied Biosystems, Monza, Italy).

Quantitative real-time PCR for genome copies was performed with LightCycler FastStart DNA master SYBR green I (Roche, Indianapolis, IN) in a total volume of 20 µl with 100 ng of template DNA and a 1 µM concentration each of AAV-specific primers (BGH F: 5'-TCTAGTTGCCAGCCATCTGTTGT-3' and BGH R: 5'-TGGGAGTGGCACCTTCCA-3' for IM injected Gunn; ITR F: 5'-

GGAACCCCTAGTGATGGAGTT-3' and ITR R: 5'-GGAACCCCTAGTGATGGAGTT-3' for IV injected Gunn). Cycling conditions consisted of 95°C for 10 minutes followed by 45 cycles at 95°C for 10 seconds, 60°C for 7 seconds, and 72°C for 20 seconds. Serial dilutions of a plasmid bearing the PCR target sequence were used as a control to determine the amounts of AAV and results were analyzed with LightCycler software version 3.5 (Roche).

Copies of hUGT1A1 mRNA in liver samples of IV injected animals were calculated by qPCR using primers that recognize either the wild-type or the codon optimized form of the hUGT1A1 (hUGT/co F: 5'-CTGCTGCTGTGTGTGCTGGGC-3' and hUGT/co R: 5'-CTGCTGCAGCTGCTGGATGGC-3'). Cycling conditions were 95°C for 10 minutes followed by 45 cycles at 95°C for 10 seconds, 70°C for 7 seconds, and 72°C for 20 seconds.

4.5 GFP expression in muscle

Following, IM injections, the following tissues were harvested from the Gunn rats: muscles (gastrocnemius, tibialis), liver, kidney, spleen, and heart. Tissues were fixed in 4% paraformaldehyde (PFA) for 24 hours. Muscle and liver specimens were embedded into paraffin blocks and sectioned into 10-µm serial sections, using a microtome, and GFP fluorescence was visualized with a Zeiss microscope. Total RNA was extracted from liver, muscle, spleen, kidney, and heart in TRIzol reagent (Invitrogen, Monza, Italy) using an RNeasy kit (Qiagen, Italy). RNA was reverse transcribed as described above.

Primers for amplification of a 95-bp fragment of GFP were as the following: GFP F, 5'-ACGACGGCAACTACAAGACC-3'; and GFP R, 5'-GTCCTCCTTGAAGTCGATGC-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. A

136-bp segment of GAPDH was amplified with the following primers: GAPDH F 5'-ATG ACTCTACCCACGGCAAG-3' and GAPDH R 5'-TAC TCAGCACCAGCATCACC-3'. Reaction conditions were as follows: reverse transcribed products were subjected to 39 cycles of amplification with 2.5U of Taq DNA polymerase in 50 μ l. After an initial denaturing cycle at 95°C for 7 minutes, subsequent cycles consisted of denaturation for 1 minute at 95°C; annealing for 1 minute at 60°C; and extension for 1 minute at 72°C.

4.6 Statistical analysis

Data are expressed as mean values \pm standard deviations in the bar and line plots. Statistical significance was computed using the Student's 2 tail test. A p-value <0.05 was considered statistically significant.

5. RESULTS

5.1 Sustained reduction of hyperbilirubinemia and excretion of conjugated bilirubin in bile and urine following muscle-directed AAV mediated gene transfer in the Gunn rats

To investigate muscle-directed AAV mediated gene therapy for CN type I, I injected Gunn rats IM with the AAV2/1-MCK-rUGT1A1 vector, encoding the UGT1A1 under the control of a muscle-specific promoter at the dose of 3×10^{12} GC/kg. The vector dose was chosen on the basis of previous studies applying a similar dose in animal models and humans (ARRUDA *et al.* 2004; MANNO *et al.* 2003). Blood samples were collected at baseline, 3 weeks after IM injections, and monthly thereafter for measurements of total serum bilirubin levels. As expected, baseline levels of total serum bilirubin in Gunn rats injected with AAV vector or saline were higher (7.95 ± 1.8 mg/dl) than the wild-type Wistar rat levels (0.91 ± 0.51 mg/dl). In Gunn rats injected with AAV vector, I observed an average 51% reduction of serum bilirubin compared to saline controls. This reduction was sustained for at least 51 weeks (**Figure 8**). Reduction in total serum bilirubin levels was not observed in saline-injected rats (**Figure 8**). The difference in serum bilirubin in AAV injected rats was statistically significant ($p < 0.05$) compared to saline control group throughout the period of study (**Figure 8**).

To monitor muscle damage after IM injections, serum CPK levels were measured at various time points (24 and 48 hours, and 1, 3, 15, 24, and 52 weeks post-injection) after IM injections and found to be not higher than saline-injected animals (**Figure 9**).

To determine whether the reduction in serum bilirubin in vector-injected animals was due to enhanced biliary excretion of bilirubin metabolites, bile was collected by cannulation of the common bile duct from AAV- and saline-injected animals at 51 weeks

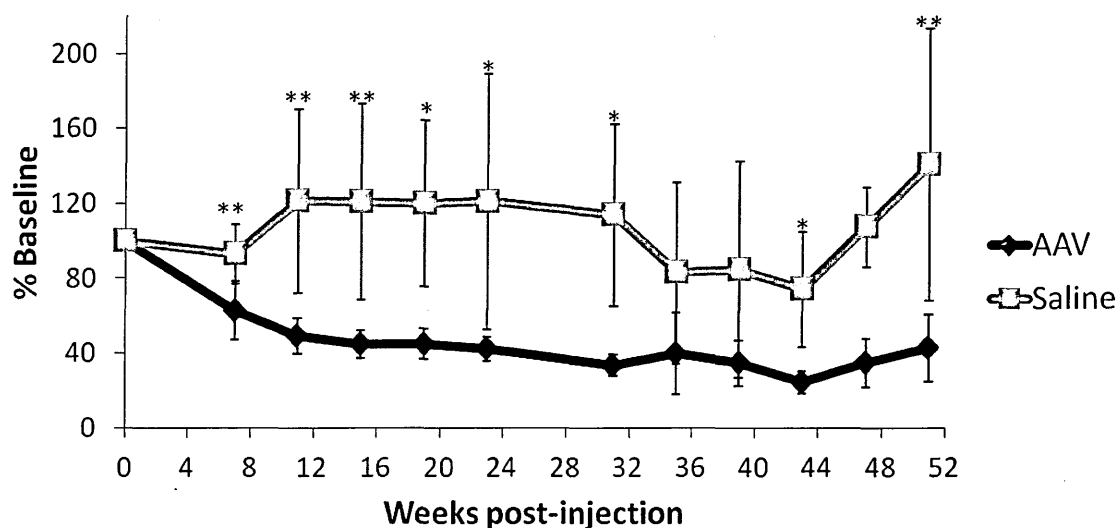


Figure 8. AAV-mediated muscle-directed gene transfer of UGT1A1 results in sustained reduction of hyperbilirubinemia in Gunn rats. Serum bilirubin levels in Gunn rats were measured at baseline and after IM injection of AAV2/1-MCK-rUGT1A1 (n = 9) or saline (n = 5). Following AAV vector IM injection, reduction of serum bilirubin was sustained for at least 51 weeks. This reduction was on average approximately 51% compared to saline injected controls (* $p<0.05$; ** $p<0.01$).

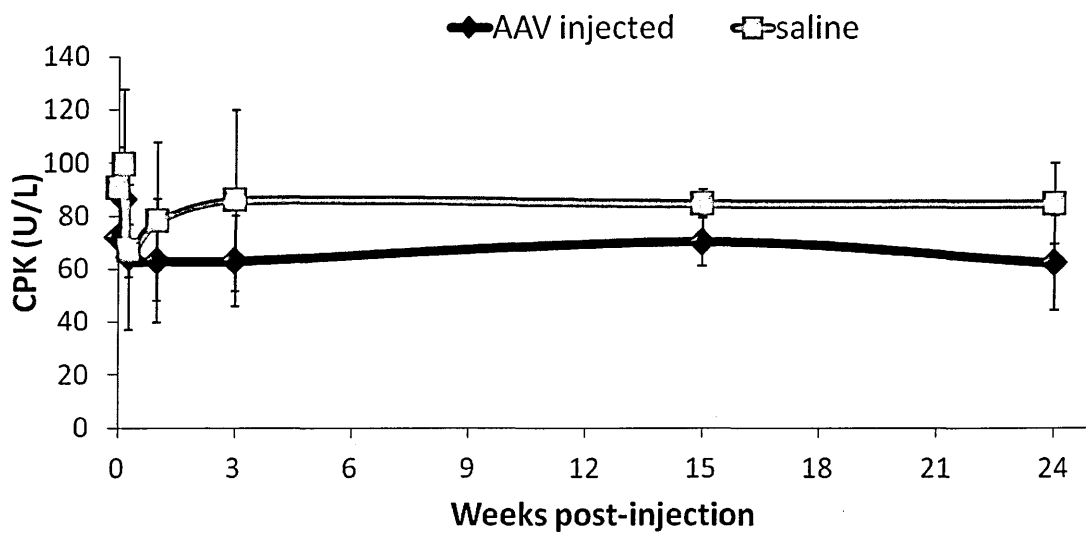


Figure 9. Evaluation of muscle damage following IM injections. Serum CPK levels were determined at various time points (24 and 48 hours, 1-3-15-24-52 weeks) post-vector administration.

post-injection. Analysis of bile (ZELENKA *et al.* 2008) showed an increase in alkali-labile bilirubin pigments in bile of AAV-injected animals compared to saline-injected controls ($p < 0.05$) (**Figure 10A**).

Similar analysis showed that AAV-injected animals also had increased excretion of bilirubin pigments in urine compared to saline-injected controls ($p < 0.05$) (**Figure 10B**) and a reduction in serum unconjugated bilirubin ($p < 0.05$) (**Figure 10C**). HPLC of bile samples showed only trace amounts, if any, of bilirubin glucuronides, but it revealed the presence of a polar metabolite peak eluting close to bilirubin diglucuronide (**Figure 11**). Although different, the absorbance of this metabolite was similar to bilirubin diglucuronide (**Figure 11**).

5.2 Expression of functionally active UGT1A1 protein in Gunn rat muscles

A subgroup of Gunn rats injected with either saline ($n = 3$) or AAV ($n = 4$) was sacrificed at 4 months post-injection to harvest muscle microsomes for determination of enzyme activity and UGT1A1 Western blotting. As controls, microsomes were prepared from muscle and liver of wild-type Wistar rats. Western blot using an anti-rUGT1A1 antibody showed a band of 55 kDa, corresponding to rUGT1A1, as shown by the band present in normal rat liver microsomes, in AAV-injected muscle microsomes but not in muscle microsomes from saline-injected controls (**Figure 12**). The presence of a band corresponding to calnexin confirmed that the purified muscle fractions corresponded to microsomes (**Figure 12**).

In the same extracts used for Western blotting, UGT1A1 activity was measured and found to be increased in AAV-injected Gunn rat muscle microsomes relative to saline injected muscles ($p < 0.05$), whereas no UGT1A1 activity was detected in liver of the same

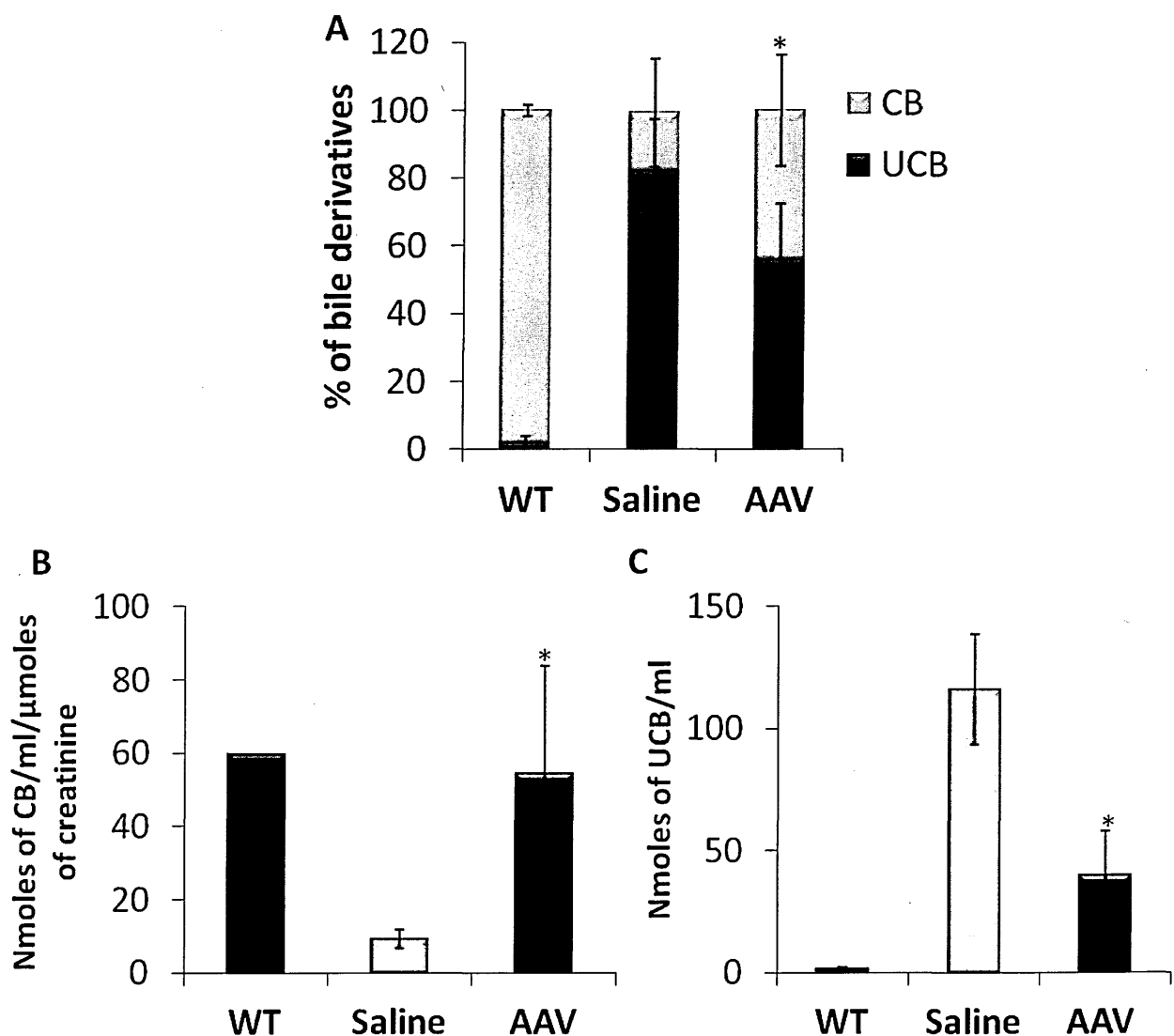


Figure 10. Bilirubin pigments in bile and urine samples. The amount of alkali-labile bilirubin pigments in the (A) bile and (B) urine of AAV-treated Gunn rats or wild-type rats, collected 1 year post-injection, was higher than in saline-injected controls ($*p < 0.05$). In bile, alkali-labile bilirubin pigments are expressed as a percentage of total bilirubin. In urine the excretion of these pigments was normalized for creatinine concentration. (C) Unconjugated bilirubin levels in serum were also found to be reduced ($*p < 0.05$). CB, conjugated bilirubin; UCB, unconjugated bilirubin; WT, wild-type.

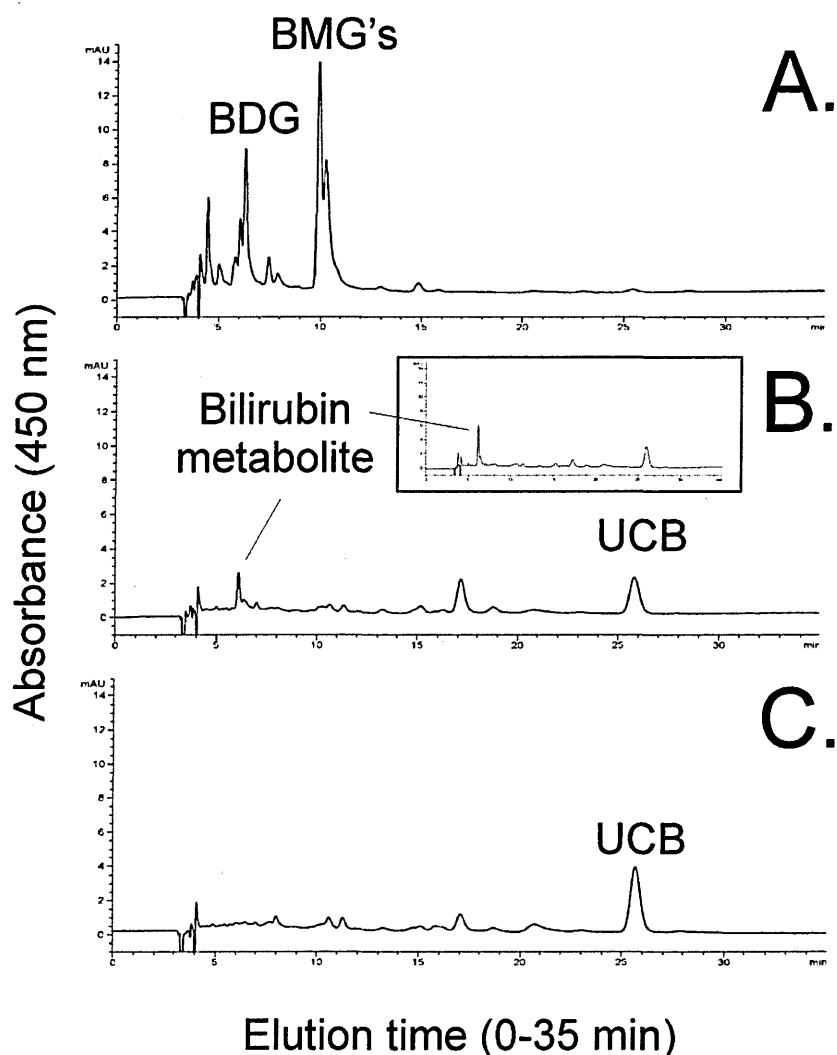


Figure 11. Bile HPLC. HPLC chromatograms of bile from wild-type rats, AAV-treated Gunn rats, and saline-injected control Gunn rats. Bile from **(A)** a wild-type rat showed strong bilirubin diglucuronide (**BDG**) and bilirubin monoglucuronide (**BMG**) peaks along with relatively minor amounts of other, unidentified peaks. Bile from **(B)** an AAV-treated Gunn rat showed a bilirubin metabolite peak, with only traces, if any, of bilirubin glucuronides along with unconjugated bilirubin (**UCB**). The metabolite peak was also evident in bile HPLC chromatogram from a different AAV treated rat and collected 4 months post-injection (shown in the inset). **(C)** Bile from a saline-injected control showed an unconjugated bilirubin peak with only a trace of other yellow pigments.

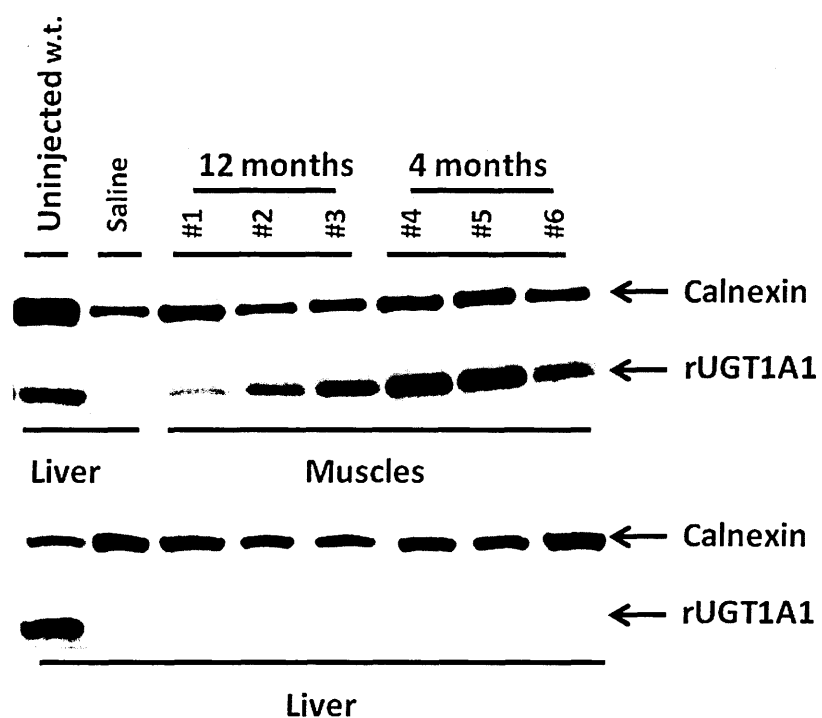


Figure 12 . UGT1A1 expression in Gunn rat muscle. Western blot analysis for rUGT1A1 protein (55 kDa) Gunn rat muscles harvested at 4 and 12 months after the IM injections of saline or with AAV2/1-MCK-rUGT1A1. As a control, a liver sample from a wild-type rat was used. Microsomal extracts were confirmed by detection of calnexin (89-kDa band).

animals (**Figure 13A**). Taken together, these results indicate that AAV-transduced muscles can express long-term functional UGT1A1 protein.

5.3 Vector biodistribution in intramuscularly injected Gunn rats

Biodistribution of AAV2/1-MCK-rUGT1A1 vector by real time PCR showed detectable vector genomes only in muscle, whereas the amounts of vector genomes detected in other organs (liver, kidney, spleen, and heart) were below the limit of detection ($<1 \times 10^2$ copies of vector genome) (**Figure 13B**).

To determine tissue specificity of transgene expression after IM injections and to rule out hepatic expression from the MCK promoter, I injected Gunn rats ($n = 3$) with AAV-MCK-GFP at the dose of 3×10^{12} GC/kg, an AAV vector expressing the reported GFP protein under the control of the MCK promoter, and sacrificed the animals at 1 month post-injection to determine tissue GFP expression and vector genome biodistribution. Injected muscles showed extensive GFP staining (**Figure 14A, a-b**), whereas no staining was detected in liver (**Figure 14A, c**). Expression of the GFP transgene driven by the MCK promoter was detected by RT-PCR in muscle, but not in liver or other tissues of the injected animals (**Figure 14B**). Together with the absence of detectable hepatic UGT1A1 protein by Western blot analysis, these data confirmed that IM injections of AAV2/1-MCK-rUGT1A1 resulted in muscle-specific expression.

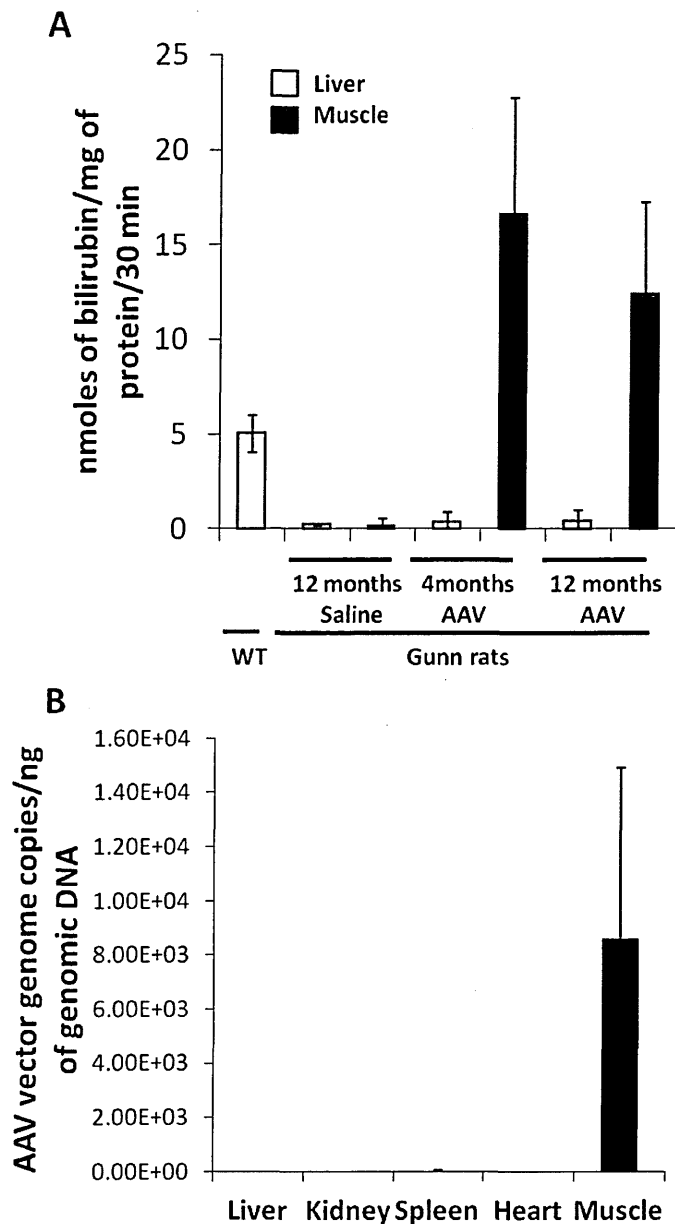


Figure 13. UGT1A1 expression in Gunn rat muscle and vector biodistribution.

(A) UGT1A1 activity (nmoles of bilirubin per milligram protein per 30 minutes) in muscle microsomes from wild-type Wistar rats ($n = 3$) and in muscles of Gunn rats injected with saline ($n = 3$) or AAV2/1-MCK-rUGT1A1, harvested at 4 and 12 months post-injection ($n = 3$ per group). The increase in enzyme activity in AAV-injected muscles was statistically significant compared to saline-injected or wild type muscle ($*p < 0.05$). (B) Analysis of AAV vector genome copies showed distribution in muscles and not in other organs ($n = 3$).

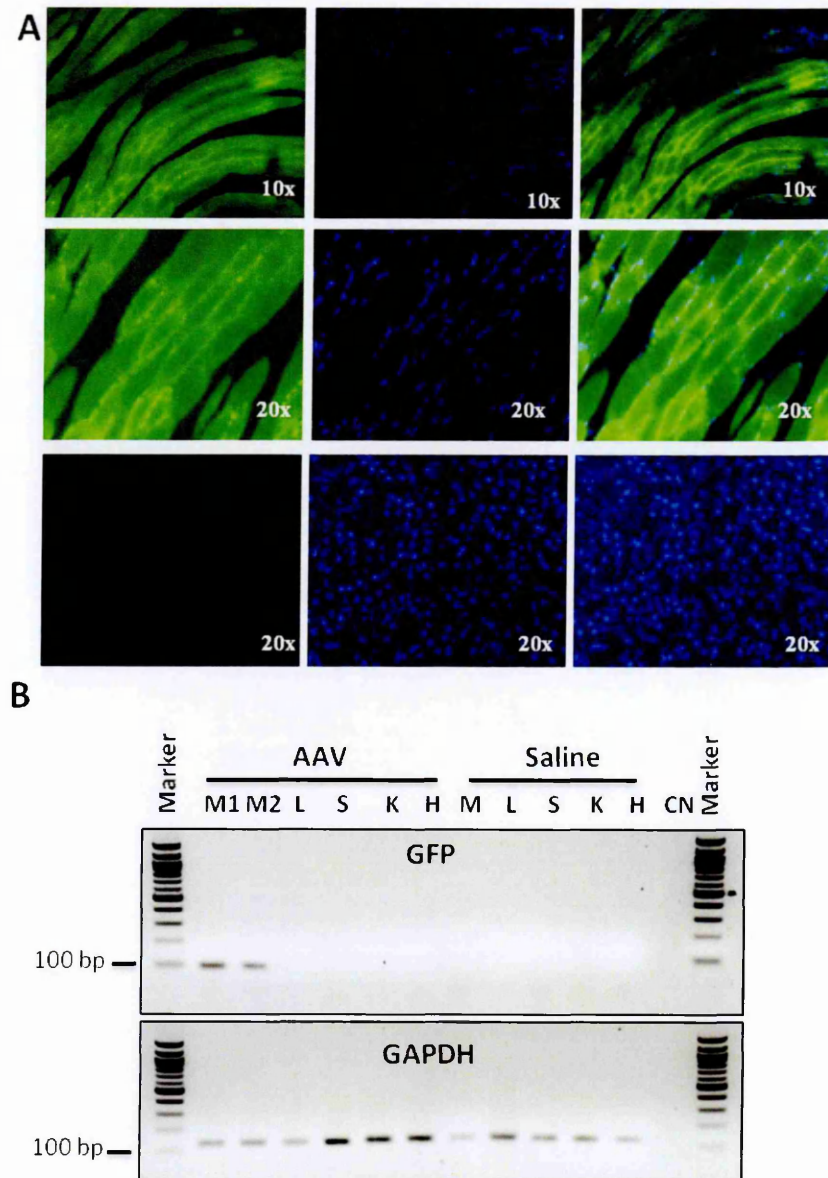


Figure 14. AAV vector muscle-specific transduction. (A) GFP positive cells were detected only in Gunn rat muscle (a-b) and not in liver (c) after IM injection of 3×10^{12} GC/kg of AAV2/1-MCK-GFP ($n = 3$). Nuclei were stained with DAPI. (B) RT-PCR showed GFP expression (95-bp fragment) in muscle but not in liver of injected animals. The 136 bp corresponding to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping control. **M1**, gastrocnemius; **M2**, tibialis; **L**, liver; **S**, spleen; **K**, kidney; **H**, heart; **CN**, negative control.

5.4 Evaluation of potency of liver specific expression cassettes *in vivo*

In the perspective of future human applications, it is important to develop a vector that has the best chances to result in clinical benefit. Therefore, I decided to investigate the potency of various expression cassettes and to determine whether a more potent expression cassette results in improved phenotypic correction in CN type I. Although not proven yet, being CN type I a non-cell autonomous disorder, it is expected that a vector expressing higher levels of UGT1A1 would result in greater reduction of hyperbilirubinemia in Gunn rats. Such a vector may allow the use of lower vector dose that will be safer and will reduce the risk of the AAV-mediated CTL response that appears to be dose-dependent (NATHWANI *et al.* 2011a). Therefore, I compared two expression cassettes including either the TBG or the LP1 promoter driving hUGT1A1 expression. These expression cassettes were then inserted within the AAV2/8 vectors that were evaluated for their efficiency in correction of hyperbilirubinemia in the Gunn rats. I decided to use the serotype 8 AAV because this vector serotype has the greatest hepatotropism in nonhuman primates and has been used successfully in hemophilia B patients (NATHWANI *et al.* 2011b). I evaluated the efficacy of AAV vectors bearing the different expression cassettes at reducing the hyperbilirubinemia *in vivo*. For these studies, I first injected 4-6 week-old female Gunn rats via tail vein with saline or with the AAV2/8-TBG-hUGT vector at the dose of 2.7×10^{13} GC/Kg. Following injections, blood samples were collected at 2 weeks and then monthly for determinations of serum bilirubin. The AAV2/8-TBG-hUGT at this high dose did not result in a significant reduction of serum bilirubin levels. Therefore, I next injected the higher dose of 7.4×10^{13} GC/Kg, that instead, resulted in 30% (5.2 mg/dl) reduction of baseline serum bilirubin by 2 weeks post-injection and 60% (2.5 mg/dl) reduction by the end of the study at 3-months post-injection (**Figure 15**). Next, I injected Gunn rats with the AAV2/8-LP1-hUGT1A1

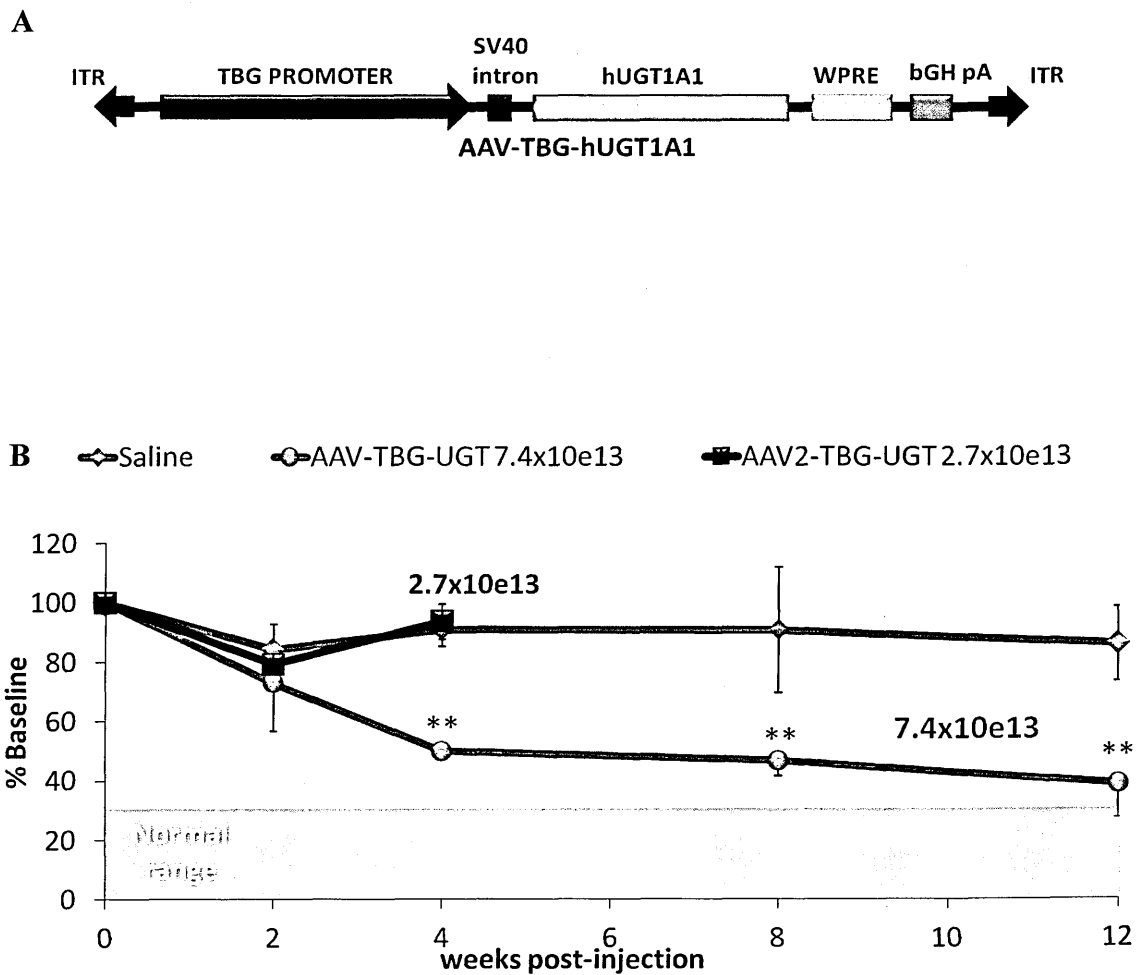


Figure 15. Serum bilirubin levels in Gunn rats after intravenous injections of AAV2/8-TBG-hUGT1A1. The vector shown in (A) presents the TBG promoter driving the expression of the UGT1A1 transgene (not drawn to scale). Following intravenous injections in Gunn rats (B), the AAV2/8-TBG-hUGT1A1 resulted in a significant reduction of hyperbilirubinemia only at the dose of 7.4×10^{13} GC/Kg (** $p < 0.01$).

vector at the dose of 2.7×10^{13} GC/Kg that resulted in 54% (2.2 mg/dl) reduction of baseline serum bilirubin by 2 weeks post-injection and this reduction was sustained for the duration of the study (**Figure 16**). Therefore, a three-fold lower dose of the AAV2/8-LP1-hUGT1A1 vector resulted in a greater reduction of bilirubin levels compared to the AAV2/8-TBG-hUGT1A1 vector. In contrast, the lower dose of 1.1×10^{13} GC/Kg did not result in reduction of serum bilirubin (**Figure 16**).

To further enhance UGT1A1 protein expression, I generated an AAV2/8 vector including the codon optimized hUGT1A1 cDNA under the control of LP1 promoter (AAV2/8-LP1-cohUGT1A1). This vector was injected into tail veins of 4-6 week-old female Gunn rats at the doses of 1.1×10^{13} GC/Kg and 1.1×10^{12} GC/Kg. The AAV2/8-LP1-cohUGT1A1 resulted in complete normalization of serum bilirubin levels corresponding to a 75% (0.76 mg/dl) reduction by 2 weeks post-injection and such correction was sustained for the entire period of observation (**Figure 17**). With a 10-fold lower dose (1.1×10^{12} GC/Kg), the reduction of serum bilirubin was 30% by 2 weeks post-injection (**Figure 17**).

Livers from injected rats were collected at the end of the study for DNA and RNA extraction to evaluate vector genome copies and hUGT1A1 expression. On total RNA extracted from livers, using specific primers that recognize the wild-type and the codon optimized hUGT1A1, I measured hUGT1A1 mRNA levels that were normalized for the number of vector genome copies given the different vector doses that were used and to take into account possible variability of vector infectivity and injection efficiency (**Figure 18B**). I found a significant increase in UGT1A1 expression in AAV2/8-LP1-hUGT1A1 injected rats compared to AAV2/8-TBG-hUGT1A1 injected rats (**Figure 18A**). A further increase in hUGT1A1 expression was found in AAV2/8-LP1-cohUGT1A1 injected rats. These

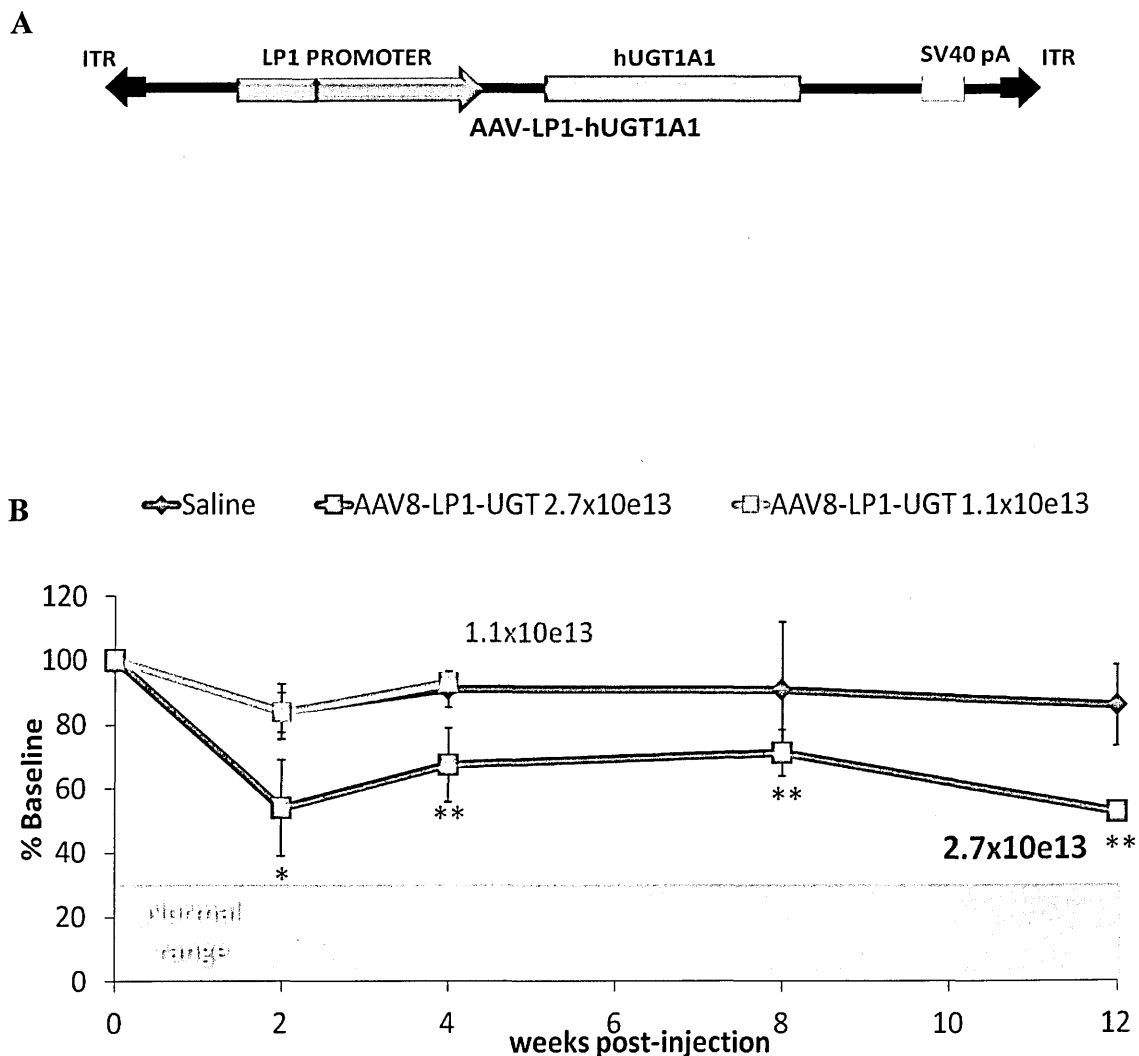


Figure 16. Serum bilirubin levels after intravenous injection of AAV2/8-LP1-hUGT1A1. The vector shown in (A) presents the LP1 promoter driving the expression of the UGT1A1 transgene (not drawn to scale). (B) The intravenous injection of AAV2/8-LP1-hUGT1A1 at the dose of 2.7×10^{13} GC/Kg resulted in 54% reduction of baseline levels by 2 weeks post-injection, whereas the injection of the same vector at the dose of 1.1×10^{13} GC/Kg did not result in significant reduction of baseline serum bilirubin levels (* $p < 0.05$; ** $p < 0.01$).

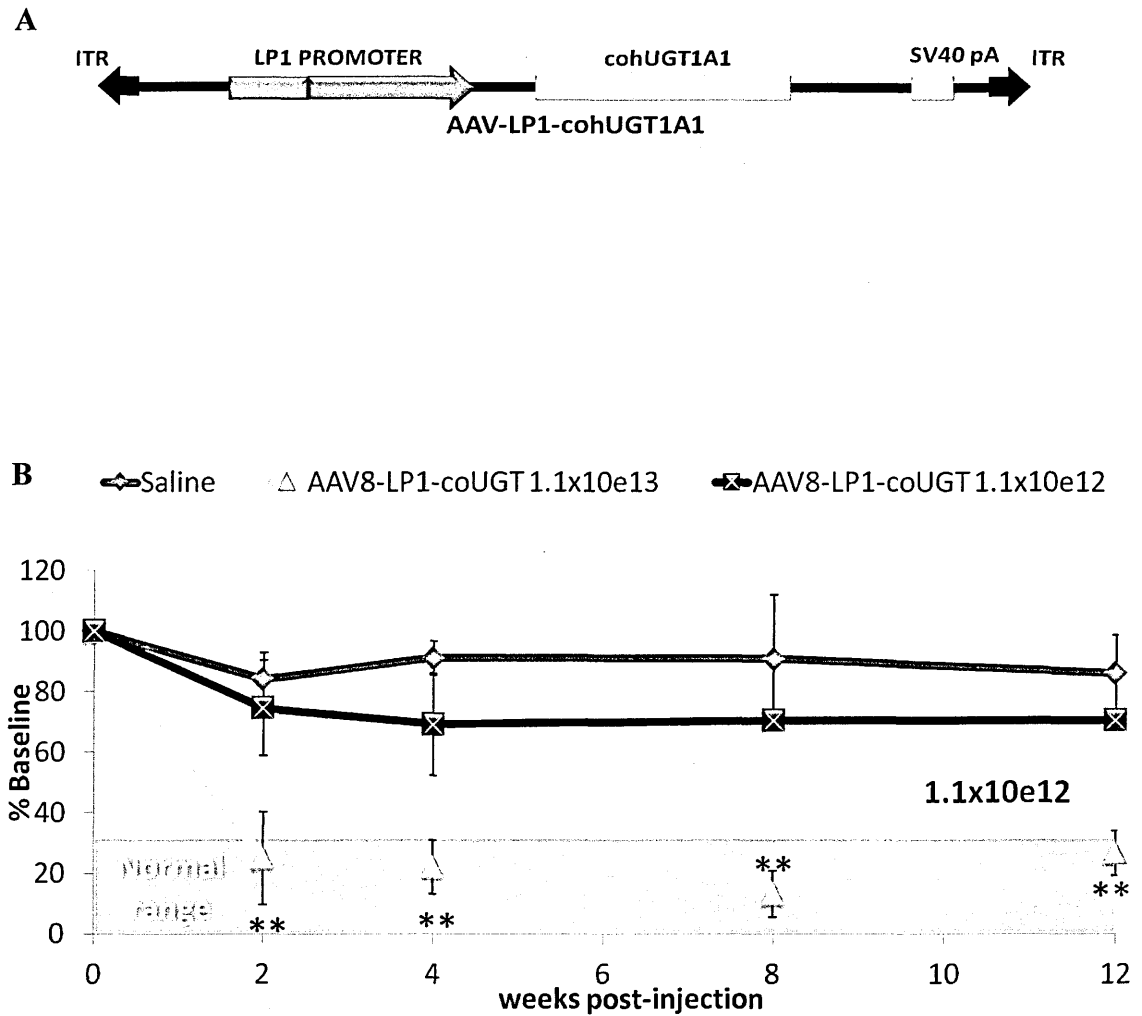


Figure 17. Serum bilirubin levels in Gunn rats injected with AAV2/8-LP1-cohUGT1A1. The vector shown in (A) presents the LP1 promoter driving the expression of the codon optimized UGT1A1 transgene (not drawn to scale). (B) Intravenous injection of AAV2/8-LP1-cohUGT1A1 at the dose of 1.1×10^{13} GC/Kg resulted in complete normalization of serum bilirubin levels by 2 weeks post-injection. The injection of the same vector at the further lower dose of 1.1×10^{12} GC/Kg resulted in 30% reduction of baseline serum bilirubin levels (** $p < 0.01$).

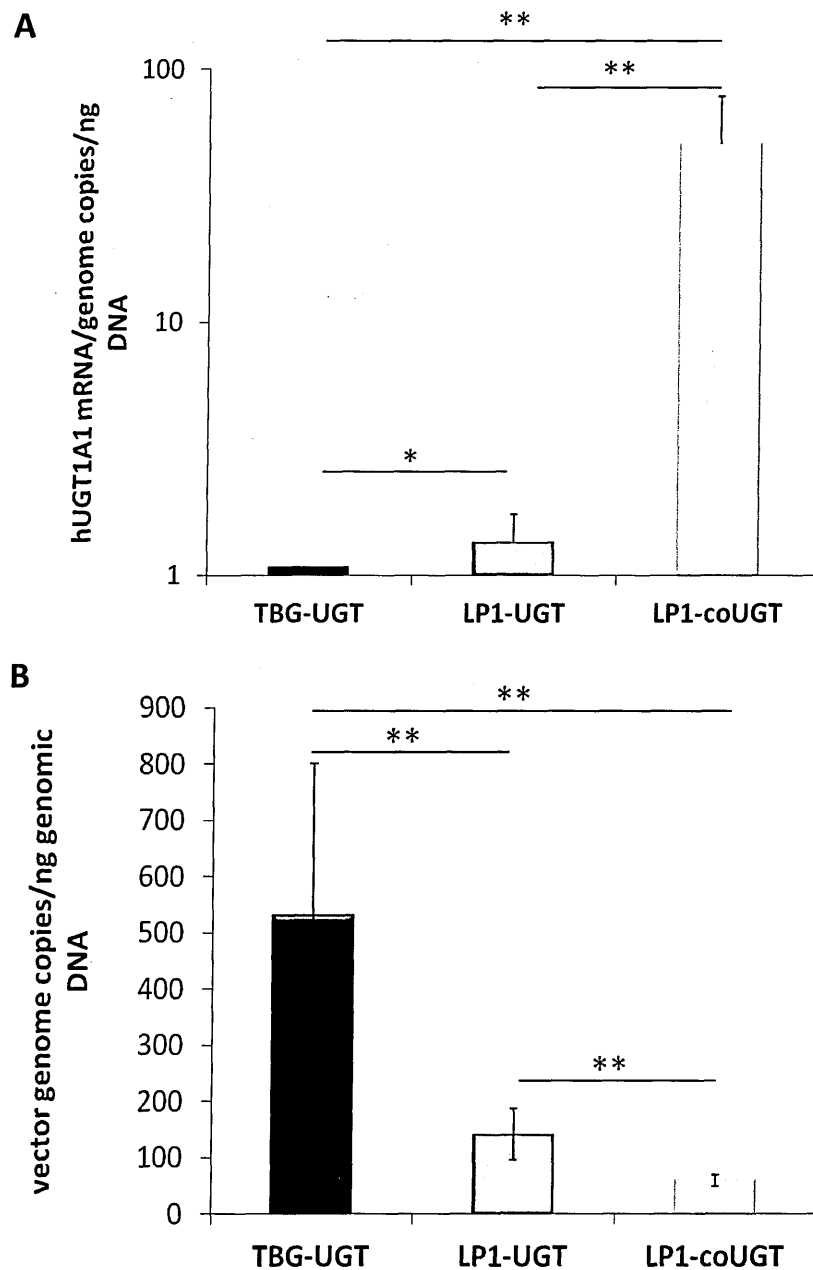


Figure 18. Expression of hUGT1A1 in liver of AAV injected Gunn rats. (A) The hUGT1A1 mRNA copies were measured in livers of Gunn rats injected with AAV2/8-TBG-hUGT1A1 (7.4×10^{13} GC/Kg), AAV2/8-LP1-hUGT1A1 (2.7×10^{13} GC/Kg), or AAV2/8-LP1-cohUGT1A1 (1.1×10^{13} GC/Kg). The hUGT1A1 mRNA levels were normalized for the amount of AAV vector genome (B) to determine the expression performance of the three injected vectors. The bars represent averages \pm SD (* $p < 0.05$; ** $p < 0.01$).

results indicate that codon optimization increases expression of hUGT1A1 in transduced liver cells and are consistent with the concept that beside the effect on translation, codon optimization affects gene expression also at the transcriptional and post-transcriptional levels by improving mRNA steady-state levels and prolonging mRNA half-lives (FATH *et al.* 2011).

6. DISCUSSION AND CONCLUSIONS

6.1 Muscle-directed gene therapy for Crigler-Najjar syndrome type I

Patients with Crigler-Najjar syndrome type I have life-threatening elevations of serum bilirubin and are currently managed with phototherapy throughout childhood and adolescence. Although effective, phototherapy is cumbersome and inconvenient, and its efficacy may diminish with age because of increased skin thickness and decreased surface-to-mass ratio (STRAUSS *et al.* 2006). Moreover, despite treatment, patients remain at risk of brain damage when intercurrent infections may increase production of bilirubin above that which can be controlled by phototherapy (STRAUSS *et al.* 2006). Therefore, patients with CN type I are often advised to consider liver transplantation, most frequently in the range of 18–25 years of age.

Muscle-directed gene therapy is attractive for CN type I because skeletal muscle is easily and safely accessible by IM injections and contains both dividing and non-dividing cells with long half-lives resulting in stable episome expression (JIANG *et al.* 2006; KOO *et al.* 2011). For this strategy to be effective in treating hyperbilirubinemia in CN, the enzyme produced in the ectopic site must be functional and expressed long term. In this thesis work, I have shown that expression of the UGT1A1 enzyme in muscle (**Figure 12**) results in long-term reduction of hyperbilirubinemia (**Figure 8**) without toxicity as demonstrated by serial CPK measurement (**Figure 9**). This finding is supported by the Western blot (**Figure 12**) and *in vitro* UGT1A1 enzyme assay (**Figure 13**), showing that muscle expresses long term a functionally active UGT1A1 protein. Bile and urine of AAV-injected animals showed increased excretion of alkali-labile bilirubin derivatives (**Figure 10**), thus suggesting that bilirubin esterified by AAV-transduced muscle cells is excreted in bile and urine. However, bilirubin mono- or diglucuronides were not detectable in bile by HPLC

and only an unidentified metabolite was detected (**Figure 10 and 11B**). This metabolite is reminiscent of the metabolite observed by Seppen and colleagues, who showed correction of hyperbilirubinemia in Gunn rats receiving transplantation of cells that do not normally express UGT1A1 but were genetically modified *ex vivo* to express UGT1A1 (SEPPEN *et al.* 1997). It is possible that UGT1A1 expressed in muscle may result in a protein that is not identical to the physiological liver-expressed protein, as previously found in the case of erythropoietin expressed at ectopic sites (LASNE *et al.* 2004; MENZEL *et al.* 2009). Whether post-translational modifications of muscle-expressed UGT1A1 play a role in the formation of this unidentified metabolite is unclear at this time.

The negligible formation of bilirubin glucuronides in the present work despite the *in vitro* detection of UGT1A1 activity in muscle microsomes may reflect inadequate formation of the necessary uridine 5'-diphosphoglucuronic acid (UDPGA) cofactor (WONG 1977). Co-expression of UDP glucose dehydrogenase (UGDH), which generates UDPGA, may further increase the therapeutic efficacy of muscle-directed gene therapy, as in the case of muscle-directed gene therapy of phenylketonuria, which requires the expression of the complete phenylalanine hydroxylase (PAH) system (PAH and BH₄-biosynthetic enzymes) to effectively clear phenylalanine from the blood (DING *et al.* 2008). Nevertheless, UGDH catalyzes the conversion of UDP-glucose to UDP-glucuronic acid that is used for production of proteoglycans, which are involved in promoting normal cellular growth and migration (AUVINEN *et al.* 2000; WANG *et al.* 2010). Therefore, overexpression of UGDH raises concern for risks of malignant transformation.

Consistent with these results, Bortolussi and colleagues have shown reduction of hyperbilirubinemia due to UGT1A1 muscle expression in *Ugt1a1*^{-/-} mice injected intraperitoneally with an AAV2/9 vector expressing human UGT1A1 under the control of

the ubiquitous CMV promoter (BORTOLUSSI *et al.* 2012). However, in those studies bilirubin glucuronide formation was demonstrated *in vitro* in muscle microsomes incubated with bilirubin and a large excess of UDPGA.

After vector intramuscular injection, AAV vector genome was detected primarily in muscle tissues with undetectable distribution to other tissues (**Figure 13B**), consistent with the conclusion that reduction of serum bilirubin was due to muscle expression of functional UGT1A1.

The main goal of gene therapy for Crigler-Najjar syndrome is prevention of brain damage due to hyperbilirubinemia; complete normalization of serum bilirubin is not required to achieve this goal. By the IM delivery, the AAV vector did not result in complete normalization of serum bilirubin levels. On average, a 51% reduction of bilirubin levels was observed as compared with saline-injected control rats (**Figure 8**). Nevertheless, such reduction is clinically highly relevant because a 30-60% decrease would result in serum bilirubin levels below 20 mg/dl in most patients (STRAUSS *et al.* 2006). Patients with CN type II with levels below this threshold are not at risk for brain damage (ARIAS *et al.* 1969). Therefore, such a reduction would result in important clinical benefit in patients with CN type I. Given the limited invasiveness of AAV-mediated muscle-directed gene therapy, this approach may be useful also for patients with CN type II, thus avoiding long-term treatment with phenobarbital.

Muscle-directed gene therapy based on pDNA delivery was previously investigated in Gunn rats (JIA and DANKO 2005a). In contrast with the pDNA study, we have demonstrated sustained correction and persistence of transgene expression after a single procedure of intramuscular injections. We also observed long-term correction of hyperbilirubinemia, whereas the study using pDNA detected immune elimination of

transfected cells caused by the presence of anti-hUGT1A1 antibodies and lymphocytic inflammation (JIA and DANKO 2005a). Possible explanations for this difference include different vectors (AAV vs. pDNA), different routes of administration (intramuscular vs. limb perfusion), different promoters (MCK vs. CMV), different transgenes (rUGT1A1 vs. hUGT1A1), or a combination thereof.

A limitation of muscle-directed gene therapy is the small number of muscle fibers that are transduced after an IM injection. However, efficient delivery methods based on limb perfusion for AAV vector delivery have been developed to allow distribution of the vector to a larger muscle mass (ARRUDA *et al.* 2010; HAURIGOT *et al.* 2010). This approach may be potentially applicable also for CN type I to permit improved phenotypic correction by transduction of a larger number of muscle cells.

AAV vectors are non integrating vectors and loss of transgene expression will occur during cell division occurring in liver of growing patients. The onset of CN type I is in the neonatal period and, thus, liver-directed gene therapy performed at that time will likely results in loss of therapeutic effect. In contrast, as shown by both small and large animal studies (SABATINO *et al.* 2007; YUE *et al.* 2008), IM injections of AAV vectors in newborns result in long-term transgene expression in muscle, a tissue that can grow by cell fusion or by increase in protein content (OTTO and PATEL 2010). Therefore, the muscle is attractive as a target organ for gene therapy for CN type I. Moreover, newborns may not mount a vigorous immune response because of their immature immune system and thus muscle-directed gene therapy in the neonatal period may be an option for patients carrying disease mutations (e.g., nonsense mutations), who are more prone to develop anti-UGT1A1 antibodies.

In summary, IM injection of AAV provided an effective, simple, and safe gene delivery method that maintained long-term functional UGT1A1 in Gunn rat muscle and resulted in clinically relevant reduction of serum bilirubin levels for at least 1 year. The reduction of serum bilirubin was associated with increased excretion of bilirubin species in bile and urine. On the basis of the HPLC spectrum, as previously shown with ectopic expression of UGT1A1 (SEPPEN *et al.* 1997), non-physiological bilirubin species were observed in Gunn rats receiving intramuscular injections of AAV vector, but their identities remain to be determined. Nevertheless, IM injections of AAV proved an effective, simple, and safe gene delivery method that maintained long-term functional UGT1A1 in Gunn rat muscle, and resulted in clinically relevant, long-term reduction of serum bilirubin levels.

6.2 Liver-directed gene therapy for CN type I with AAV2/8

Peripheral vein infusion of self-complementary AAV2/8 vector encoding FIX under the control of a liver-specific promoter resulted in FIX transgene expression at levels sufficient to improve the bleeding phenotype in patients with hemophilia B (NATHWANI *et al.* 2011b). Although immune-mediated clearance of AAV-transduced hepatocytes remains a concern and was eventually controlled with a short course of glucocorticoids, this clinical trial may pave the way towards applications of liver-directed gene therapy for a wide range of inborn errors of liver metabolism, including CN type I.

AAV vectors have several attributes that make them highly suitable for liver-directed gene therapy (NATHWANI *et al.* 2009). Studies in mice shown that at high vector doses, AAV2 can transduce up to 5-10% of the hepatocytes (NAKAI *et al.* 2002) whereas the novel AAV serotypes, particularly AAV8, have improved hepatic transduction efficiency (DAVIDOFF *et al.* 2005).

A previous study investigated the efficiency of various AAV serotypes in the Gunn rats and found a reduction of serum bilirubin with all three AAV serotypes tested (AAV1, AAV6, and AAV8) (SEPPEN *et al.* 2006). AAV1 was found to be the most effective and superior to AAV8. These results highlight species specificity as an important factor for AAV serotype transduction efficiency. Nevertheless, AAV8 remains the most attractive vectors considering the higher transduction efficiency achieved in nonhuman primate livers. This animal model is in fact considered more adequate to predict the human outcome. AAV8 is also attractive because compared to other serotypes it results in more rapid increase of transgene levels, less profound immune responses, and more limited biodistribution (NATHWANI *et al.* 2007). The previous study by Seppen *et al.* used AAV vectors with the ubiquitous CMV promoter. This promoter is not acceptable for human clinical trial because it results in higher likelihood of antibody response against the transgene product and it is also silenced over time in animal models (PASTORE *et al.* 1999). A critical factor in gene therapy is the requirement of sustaining expression of a therapeutic gene at an appropriate amount, but also expression in specific tissues. For this reason, promoter selection is particularly important.

During my thesis work, I compared two different expression cassettes within the AAV2/8 vector with the goal of identifying the most effective vector for liver-directed gene therapy of CN type I. Moreover, by comparing expression cassettes of different potency, I also investigated whether phenotypic correction is indeed affected by the transgene levels. Although it is predicted that being a non-cell autonomous disorder, higher transgene levels would result in improved phenotypic correction, this hypothesis has not been tested before.

The expression cassettes I used for my studies had either the TBG or the LP1 promoter. The TBG is a 54-kDa glycoprotein that is primarily active in hepatocytes and the TBG promoter has been frequently used to regulate various therapeutic genes in the liver (BISH *et al.* 2011; CARRILLO-CARRASCO *et al.* 2010; COTUGNO *et al.* 2011; GAO *et al.* 2002). Recent studies (YAN *et al.* 2012) comparing relative activities of several constitutive (CMV, phosphoglycerate kinase, EF1 α ,) and tissue-specific (apolipoprotein E, cytochrome P450 2E1) promoters have shown that the TBG promoter results in stable and high levels of transgene expression *in vivo*. The LP1 is a synthetic promoter that consists of the core domains from the human apolipoprotein hepatic control region and the human alpha-1-antitrypsin gene and has been used in several preclinical studies and in the hemophilia B AAV gene therapy trial (NATHWANI *et al.* 2007; NATHWANI *et al.* 2006; NATHWANI *et al.* 2011a).

The *in vivo* studies demonstrated that Gunn rats injected intravenously with the vector expressing the codon optimized hUGT1A1 exhibited higher UGT1A1 levels when normalized for the vector genomes (**Figure 18**). Through a head-to-head comparison of the vectors encoding the wild-type hUGT1A1, I also observe higher transgene expression with the LP1 promoter compared to the TBG promoter (**Figure 18**). Importantly, the AAV8 vector expressing the codon optimized hUGT1A1 cDNA resulted in complete normalization of serum bilirubin levels at low vector doses (**Figure 17**). Clinical trials performed in hemophilia B patients using AAV vectors for liver-directed gene therapy have shown that there is a direct relationship between the AAV dose and the levels of hepatotoxicity as a consequence of an adaptive cellular immune response against the transduced hepatocytes (MANNO *et al.* 2006; MINGOZZI *et al.* 2007; NATHWANI *et al.* 2011b). Reducing the AAV dose may help to evade this immune response and therefore,

vector harboring more potent expression cassette described herein may be useful for achieving therapeutic effect with lower vector doses.

Several studies have shown the consequences of codon usage on gene expression in mammalian cells (GUSTAFSSON *et al.* 2004). Gene expression in mammalian cells can be significantly enhanced by substituting rare codons with more frequent codons (WELLS *et al.* 1999; ZOLOTUKHIN *et al.* 1996). Each organism has its preferred choice of nucleotide usage to encode any particular amino acid. Besides variations between species, codon usage also differs among human tissue cells (PLOTKIN *et al.* 2004) and housekeeping genes are usually associated with higher GC-content. Differences in tissue specific expression of individual tRNA species and the relative abundance of tRNA-iso-acceptors strongly correlate with codon usage of genes highly expressed in specific tissues (DITTMAR *et al.* 2006).

The codon optimization process is based on modification of the gene sequence according to the tRNA abundance in a specific species. We optimized the human sequence of hUGT1A1 for the expression in *Homo sapiens*. The increase in expression we observed in the Gunn rats could be related to the similar codon usage between *Homo sapiens* and *Rattus norvegicus* (Figure 19).

In summary, our study shows that AAV2/8 vector with codon optimized UGT1A1 gene under the control of the hepatocyte-specific LP1 promoter results in improved and sustained correction of hyperbilirubinemia in Gunn rats. Taken together, these data demonstrate the development of an optimal expression cassette for liver-directed gene therapy of CNH and form the preclinical basis for the development of a gene therapy trial for this severe disorder.

<i>Homo sapiens</i> [gbpri]: 93487 CDS's (40662582 codons)				
fields: [triplet] [frequency: per thousand] ([number])				
UUU 17.6(714295)	UCU 15.2(618711)	UAU 12.2(495699)	UGU 10.6(430311)	
UUC 20.3(824692)	UCC 17.7(718892)	UAC 15.3(622407)	UGC 12.6(513028)	
UUA 7.7(311881)	UCA 12.2(496448)	UAA 1.0(40285)	UGA 1.6(63237)	
UUG 12.9(525688)	UCG 4.4(179419)	UAG 0.8(32109)	UGG 13.2(535595)	
CUU 13.2(536515)	CCU 17.5(713233)	CAU 10.9(441711)	CGU 4.5(184609)	
CUC 19.6(796638)	CCC 19.8(804620)	CAC 15.1(613713)	CGC 10.4(423516)	
CUA 7.2(290751)	CCA 16.9(688038)	CAA 12.3(501911)	CGA 6.2(250760)	
CUG 39.6(1611801)	CCG 6.9(281570)	CAG 34.2(1391973)	CGG 11.4(464485)	
AUU 16.0(650473)	AUC 13.1(533609)	AUA 17.0(689701)	AGU 12.1(493429)	
AUA 7.5(304565)	AUA 15.1(614523)	AAA 24.4(993621)	AGA 12.2(494682)	
AUG 22.0(896005)	ACG 6.1(246105)	AAG 31.9(1295568)	AGG 12.0(486463)	
GUU 11.0(448607)	GCU 18.4(750096)	GAU 21.8(885428)	GGU 10.8(437126)	
GUC 14.5(588138)	GCC 27.7(1127679)	GAC 25.1(1020595)	GGC 22.2(903565)	
GUA 7.1(287712)	GCA 15.8(643471)	GAA 29.0(1177632)	GGA 16.5(669873)	
GUG 28.1(1143534)	GCG 7.4(299495)	GAG 39.6(1609975)	GGG 16.5(669765)	
Coding GC 52.27% 1st letter GC 55.72% 2nd letter GC 42.54% 3rd letter GC 58.55%				

<i>Rattus norvegicus</i> [gbrod]: 15151 CDS's (6981149 codons)				
fields: [triplet] [frequency: per thousand] ([number])				
UUU 16.5(115498)	UCU 14.8(103148)	UAU 11.6(80999)	UGU 9.8(68418)	
UUC 23.1(161568)	UCC 17.8(124290)	UAC 17.1(119192)	UGC 11.9(82751)	
UUA 5.9(41454)	UCA 10.9(76435)	UAA 0.6(4473)	UGA 1.2(8137)	
UUG 12.8(89203)	UCG 4.4(30458)	UAG 0.5(3637)	UGG 13.2(91958)	
CUU 12.5(87332)	CCU 17.4(121358)	CAU 9.5(66664)	CGU 5.0(34788)	
CUC 20.4(142093)	CCC 18.0(125645)	CAC 14.9(104027)	CGC 9.8(68486)	
CUA 7.6(52955)	CCA 16.1(112364)	CAA 11.1(77366)	CGA 6.8(47207)	
CUG 41.1(286626)	CCG 6.3(43713)	CAG 33.8(235764)	CGG 10.9(76123)	
AUU 15.3(106736)	AUC 12.9(90372)	AUA 15.1(105212)	AGU 11.8(82659)	
AUA 6.9(48257)	AUA 15.3(106544)	AAA 21.5(150018)	AGA 11.2(77865)	
AUG 23.1(161552)	ACG 6.2(43237)	AAG 35.1(245259)	AGG 11.8(82391)	
GUU 10.4(72270)	GCU 19.7(137491)	GAU 20.8(146211)	GGU 11.4(79443)	
GUC 16.2(113143)	GCC 27.1(189524)	GAC 28.0(195508)	GGC 21.9(152582)	
GUA 7.2(50048)	GCA 15.6(109203)	GAA 26.9(187810)	GGA 16.6(115968)	
GUG 30.0(209458)	GCG 6.9(47869)	GAG 41.3(288345)	GGG 15.6(108657)	
Coding GC 52.59% 1st letter GC 55.66% 2nd letter GC 41.70% 3rd letter GC 60.40%				

Figure 19. Codon usage in *Homo sapiens* and *Rattus norvegicus*.

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